Central regulation of melanotrope cells in *Xenopus laevis*

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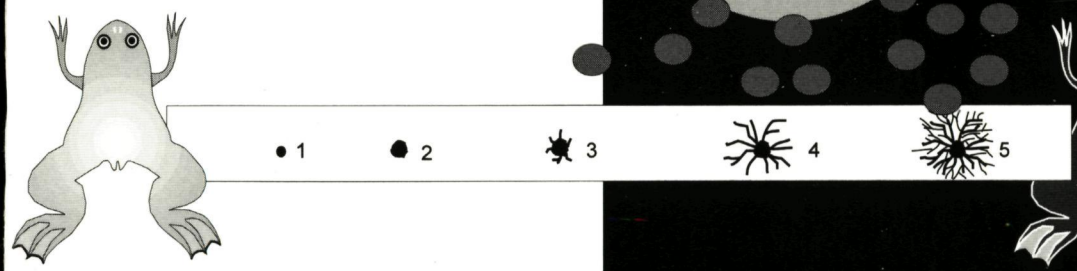
RIENK TUINHOF



CENTRAL REGULATION OF MELANOTROPE CELLS IN *XENOPUS LAEVIS*

CENTRAL REGULATION OF MELANOTROPE CELLS

IN *XENOPUS LAEVIS*

een wetenschappelijke proeve op het gebied van
de Natuurwetenschappen

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen
op maandag 11 september 1995,
des namiddags te 3.30 uur precies

door

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geboren te Irnsum

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Oan al dejinge dy mij
sa nei oan't hert lizze

Printed by Copyprint 2000, Enschede, The Netherlands

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Tuinhof, Rienk

Central regulation of melanotrope cells in *Xenopus laevis*
/ Rienk Tuinhof. - [S.l. : s.n.]. - Ill.
Thesis Katholieke Universiteit Nijmegen. - With ref. - With summary in Dutch.
ISBN 90-9008527-0
Subject headings: *Xenopus laevis* / melanotrope cells / central regulation / background adaptation

CENTRAL REGULATION OF MELANOTROPE CELLS IN *XENOPUS LAEVIS*

CONTENTS

GENERAL INTRODUCTION

CELLULAR COMMUNICATION

To survive it is essential for a multicellular organism to be able to adapt to changing conditions of the environment. Important for this adaptation is the perception of information about the environment and about the internal state of the organism. Processing of this information enables the regulation of correct functioning of cells, tissues, organs and the organism as a whole. Two important organ systems involved in the transduction of external signals (light, temperature, food, *etc.*) into commands to internal effector systems (*e.g.* muscles and glands) are the nervous and the endocrine system. These two systems differ in the pathways they deliver messages and the structure of their messenger molecules.

The nervous system consists of nerve cells, or neurons, that are specialized to convey rapidly, and very precisely, signals over long distances (see Kandel et al., 1991). The neurons (Fig. 1) consist of a cell body, or soma, from which long processes extend, namely several dendrites (receiving input from other neurons) and usually one long process, the axon, which provides the connection with the target (other neurons, muscles or glands). Neurons respond to stimuli with an electrical response, the action potential, which rapidly moves along the axon membrane towards the axon terminal. The axon terminals generally form nearby-contacts with the cell membrane of the target cell, the so-called synapses. Chemical messengers or neurotransmitters synthesized in the perikaryon, are transported along microtubuli (Oakley and Oakley, 1995) through the axon towards the synapse, where they are stored. In the classical view one neuron produces only one neurotransmitter (Dale, 1935), such as acetylcholine, glutamate, GABA and mono-

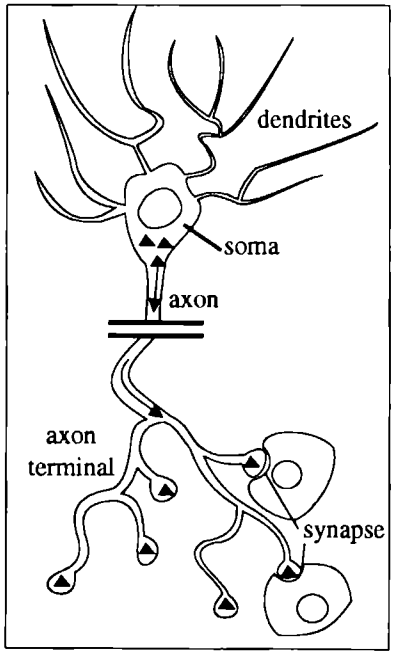


Fig. 1. Schematic drawing of the basic element of the nervous system, the neuron.

amines (adrenaline, noradrenaline, dopamine, serotonine or histamine); also neuropeptides (*e.g.* neuropeptide Y and thyrotropin releasing factor, TRH) can act as neurotransmitters. More recently it was shown that neurons can produce, store and release more than one neurotransmitter at the same time (Fig. 2; *e.g.* Lundberg and Hökfelt, 1983; Hökfelt et al., 1984, 1986). A nerve impulse can elicit the release of the messengers from the synapse via exocytosis into the synaptic cleft. From here the messengers diffuse to their target to reach specific receptors on the postsynaptical cell membrane which may induce a cell-specific, rapid effect.

The second information transducing system, the endocrine system, acts slower, as it mediates its effects via the secretion of another type of messenger, the hormones (*e.g.* peptides, glycoproteins and steroids). Endocrine cells do not contact their targets directly but act over long distances by releasing hormones into the circulation. Generally, the effects of hormones last longer than those of neurotransmitters.

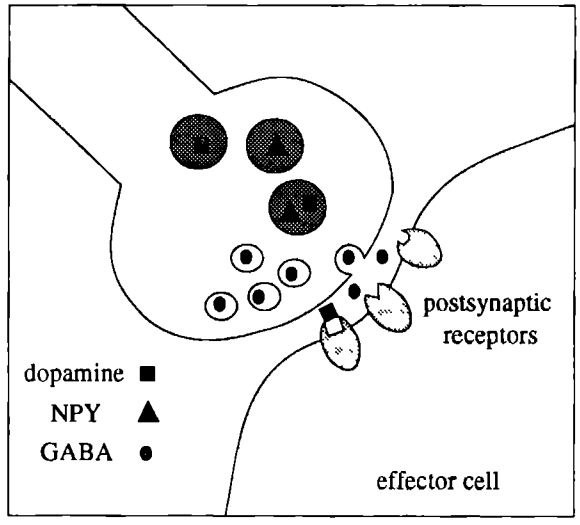


Fig. 2. Inhibitory synapse containing multiple neurotransmitters that are released to deliver the message to the postsynaptic receptors of the effector cell

In the hypophysis the nervous and endocrine system cooperate in the control of peripheral targets, *e.g.* in the regulation long-term physiological processes like growth, development, reproduction and adaptation. Neurons in the hypothalamus influence the activity of the hypophysis in two different ways: 1/ by direct synaptic contacts, and 2/ by neurohormones released from the median eminence or the pars nervosa of the hypophysis ("neurosecretion"; Etkin, 1962a,b; Bargman et al., 1967; Iturriza, 1969; Perryman, 1974; Hadley and Bagnara, 1975; Weatherhead, 1983).

An interesting type of cooperation between the nervous and endocrine

systems is found in the hypophysis of the South African clawed toad, *Xenopus laevis*, where the melanotrope cells of the pars intermedia of the hypophysis, which play a pivotal role in the complex process of background adaptation, are controlled by various neurotransmitters and neurohormones.

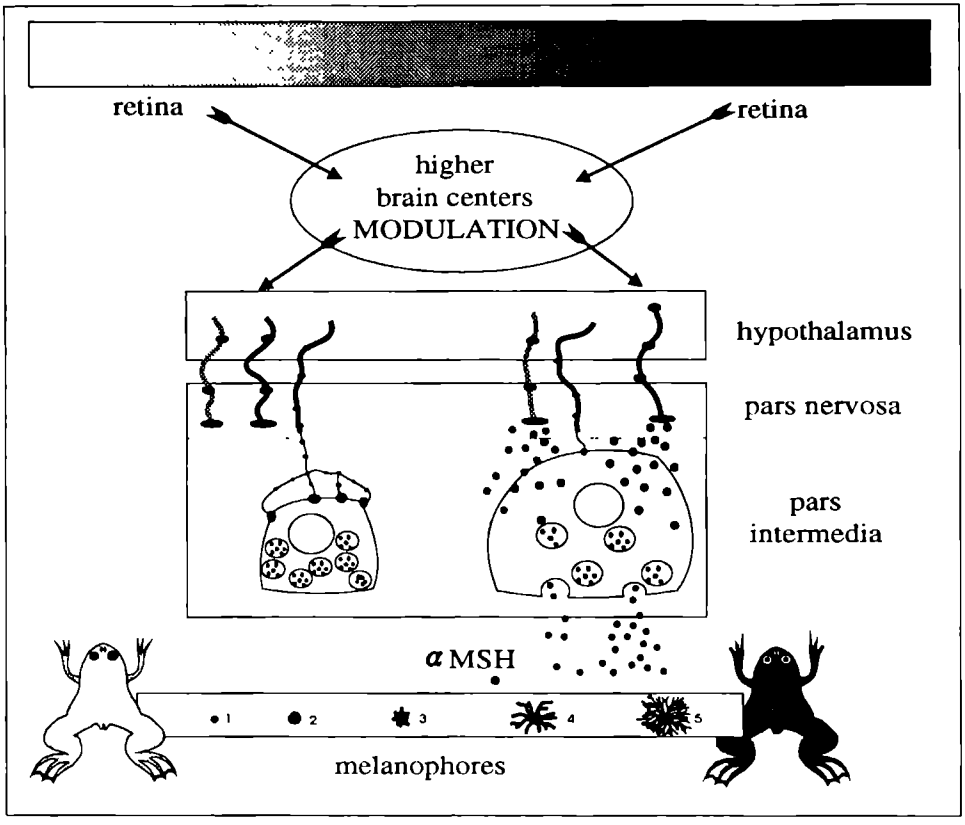


Fig. 3. The regulatory pathway of the process of background adaptation in *Xenopus laevis*. The information of light intensity of background (white to black) is transmitted by the retina via higher brain centers to the hypothalamus The hypothalamus regulates the activity of the α-MSH producing and secreting cells in the pars intermedia of the hypophysis The presence of α-MSH in the circulation causes dispersion of the black pigment, melanin in the dermal melanophores leading to darkening of the skin Absence of α-MSH leads to aggregation of melanin and subsequently, paling of the skin

BACKGROUND ADAPTATION

Optimal functioning of an organism requires an adequate adaptation to changes in environmental conditions, such as adaptation of skin color. Skin

color adaptation can serve different purposes, *e g* camouflage or, in contrast, attraction of attention, delivery of warning signals or the protection of the skin against excessive solar heat In lower vertebrates (reptiles, amphibians and fish), a variety of external and internal stimuli can cause changing of skin color, such as light intensity, temperature, psychic stimuli, internal rhythms and humidity (Waring, 1963, Terlou et al , 1974)

The permanently aquatic toad, *Xenopus laevis*, is capable of matching the color of its skin to the light intensity of its surroundings (Fig 3) The sensory information concerning the light intensity is perceived by the retina, translated into electrical and chemical signals, and transmitted to different visual centers in the central nervous system (Lázar and Székely, 1969, Lázár, 1978, Levine, 1980, Tóth et al , 1980) After modulation in the brain, the information is passed on to hypothalamic centers that subsequently stimulate or inhibit the production, storage and secretion of melanotropic hormones by particular endocrine cells in the hypophysis These melanotrope cells produce α-melanophore stimulating hormone (α-MSH), which evokes after transport via the circulation, a blackening of the skin by stimulating the dispersion of black pigment in dermal melanophores

XENOPUS LAEVIS AND BACKGROUND ADAPTATION AS RESEARCH MODEL

The neuroendocrine regulatory mechanism of background adaptation in the brain of *Xenopus laevis* is a suitable object for anatomical and physiological research of the way the nervous and endocrine system cooperate in the control of important body functions Firstly, in contrast to the situation in mammals the function of the melanotrope cells in *Xenopus* is well known Secondly, the regulatory mechanism of background adaptation represents a system in which both the input (light intensity) and the output (release of α-MSH) can be readily quantified The different levels in the regulatory pathway, namely the neural integration, the neuroendocrine transmission, the receptor-second messenger interactions and the secretory mechanism, all offer the possibility of multidisciplinary investigations to gain a better insight into the nature of biological signal transduction mechanisms in general

For anatomical research the major advantages of the melanotrope

regulatory system of *Xenopus* are that, 1/ in comparison to other amphibians, the brain of *Xenopus* does not contain abundant melanin, which would obscure microscopic observations, 2/ the relative ease to raise and maintain the aquatic *Xenopus* under laboratory conditions and 3/ the availability of an accurate time-table from the larval stages into adulthood (Nieuwkoop and Faber, 1967) makes this animal especially suitable for developmental investigations.

THE MELANOTROPE CELLS

As α-MSH plays a crucial role in the regulation of background adaptation in amphibians, the melanotrope cells of *Xenopus* have been studied extensively over the past two decades, physiologically as well as molecular biologically. The peptide is derived from the precursor protein proopiomelanocortin (POMC; see Weatherhead, 1983; Martens, 1987; Martens et al., 1989; Deen et al., 1991). Especially in animals that are placed on a dark background, the transcriptional activity of the POMC-genes is very high (Martens, 1987) resulting in an increased biosynthesis of POMC-derived peptides. Crucial roles in the proper execution of the secretory pathway are played by the proteins 7B2 and PC2. 7B2 is a molecular chaperone that mediates correct protein folding and assembly by interaction with prohormone convertase PC2, which is involved in cleavage of POMC. In the pars intermedia the expression patterns of 7B2 and PC2 are similar to that of POMC (Ayoubi et al., 1991; Braks and Martens, 1994). On a black background, the high expression of POMC results in an raised production of α-MSH, which is secreted into the vascular system and transported throughout the body. After reaching the skin, α-MSH causes a dispersion of the black pigment melanin, in the skin melanophores, which darkens the color. The high activity of the melanotrope cells under black background conditions is reflected by a large hypertrophic pars intermedia (Pehleman, 1967; De Rijk et al., 1990a,b; Ayoubi et al., 1991). In animals that are placed on a white background, the transcription activity of POMC and the subsequent production and release of α-MSH are reduced, resulting in a hypotrophic pars intermedia. The absence of α-MSH in the circulation causes an aggregation of melanin to a perinuclear location within the dermal melanophores giving the skin a pale appearence (Waring, 1963; Jenks and Van Zoest, 1990; Jenks et al., 1988,1993).

REGULATION OF MELANOTROPES

Hormone secretion from the hypophysis in vertebrates is regulated by stimulatory and inhibitory neurotransmitters originating in the hypothalamus (*e.g.* Vale et al., 1973; Terlou et al., 1974; Jenks et al., 1993). Removal of the hypophysis of *Xenopus* results in an increased secretion of α-MSH, due to the loss of hypothalamic neural inhibition (Hadley and Hruby, 1977). That the hypothalamus is a part of the pathway between sensory light perception and α-MSH secretion, appears also from the fact that transsection of the hypothalamic pathway to the hypophysis results into a darkening of the skin even when the animals are kept on a white background (Etkin and Sussman, 1961; Jørgensen and Larsen, 1963; Enemar and Falck, 1965), suggesting that the hypothalamus predominantly exerts an inhibitory effect on the melanotrope cells in amphibians (Etkin, 1962a,b; Jørgensen and Larsen, 1963).

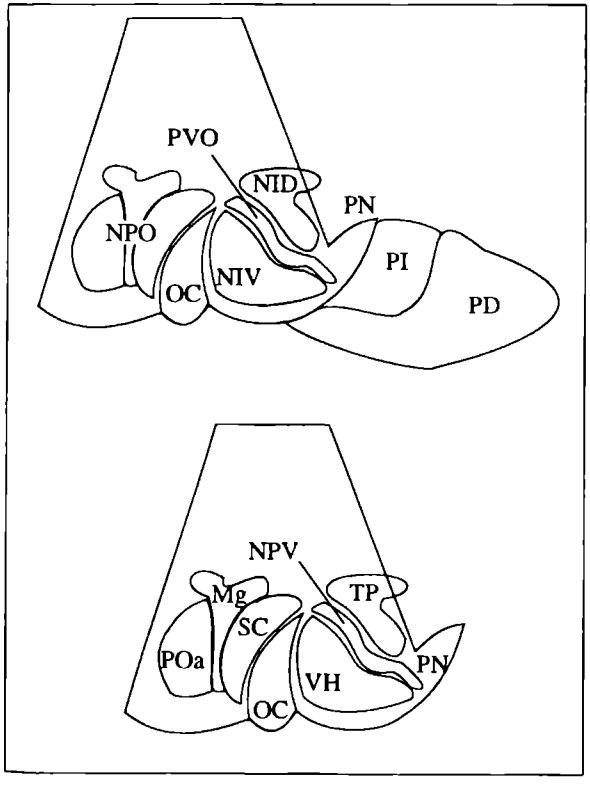


Fig. 4. Diagram of a sagittal section from the brain of *Xenopus laevis*. In the upper drawing the nomenclature is adopted from Wada et al. (1980) From rostral to caudal the hypothalamus and hypophysis contain *NPO*, preoptic nucleus; *OC*, optic chiasm; *NIV*, ventral infundibular nucleus, *PVO*, nucleus of the paraventricular organ, *NID*, dorsal infundibular nucleus, *PN*, hypophysis, pars nervosa, *PI*, hypophysis, pars intermedia, *PD*, hypophysis, pars distalis

The nomenclature used in this thesis is adopted from Neary and Northcutt (1983) and is shown in the lower drawing *POa*, anterior preoptic nucleus, *Mg*, magnocellular nucleus, *OC*, optic chiasm, *PN*, hypophysis, pars nervosa; *SC*, suprachiasmatic nucleus; *VH*, ventral infundibular nucleus; *NPV*, nucleus of the paraventricular organ, *TP*, posterior tubercle.

Pharmacological and morphological data furthermore suggested that this inhibition would be of catecholaminergic nature (Enemar and Falck, 1965, Dierst-Davies et al , 1966, Pehlemann, 1967, Nakai and Gorbmann, 1969, Oshima and Gorbmann, 1969, Bower et al , 1974) Application of 6-hydroxydopamine, a neurotoxin that destroys catecholaminergic innervation, resulted in blackening of the skin (De Volcanes and Weatherhead, 1976a,b) Extirpation of the preoptic nucleus (NPO, Fig 4) had no effect on the ability of the animal to adjust its skin color when it was transferred from a black to a white background (Dierickx, 1967) This finding led to the conclusion that the catecholamine-producing neurons, which are responsible for the inhibition of the melanotropes are located in the hypothalamus, in an area caudal to the optic chiasm Indeed the postoptic hypothalamic region of *Rana pipiens* is very sensitive to electrical stimulation, resulting in dispersion of the dermal melanophores (Dierst and Ralph, 1962) Using the light microscopical formaldehyde-induced fluorescence method, presumed dopaminergic neurons were identified in the caudal (postoptic) hypothalamus in the paraventricular organ (PVO, Fig 4) and in the dorsal infundibular nucleus (NID, Fig 4, Terlou and Ploemacher, 1973, Terlou and Van Straaten, 1973, Terlou et al , 1974, nomenclature by Wada et al , 1980) The dopaminergic neurons of the PVO send protrusions into the third ventricle (Peute, 1971, Peute and Van Oordt, 1974) These liquor-contacting neurons of the PVO and/or the dopamine-containing neurons in the NID were proposed as the origin of the aminergic hypothalamo-hypophysial tract that could be seen running through the median eminence via the pars nervosa into the pars intermedia (Terlou and Ploemacher, 1973) After blinding or removal of the telencephalon and diencephalon, the skin of black background-adapted *Xenopus* tadpoles remained black when the animals were placed on a white background Immersion of such larves into a reserpine solution caused a strong decline in the melanophore index (Goos, 1969), indicating the reserpine-induced release of dopamine acting on the melanotropes Removal of the NID and the rostral half of the PVO did not affect α-MSH secretion However, destruction of the caudal part of the PVO caused a strong increase of α-MSH release and subsequent blackening of the skin (Terlou et al , 1974), suggesting that the caudal part of this nucleus is responsible for the inhibition of the melanotrope cell activity (Goos, 1978)

More recently, however, preoptic centers, namely, in *Rana temporaria* the anterior preoptic nucleus (Prasado Rao, 1982), in *Bufo japonicus* the preoptic recess organ (Kato et al., 1992) and in *Rana catesbeiana* (Carr et al., 1991) the dopaminergic suprachiasmatic and dorsal infundibular

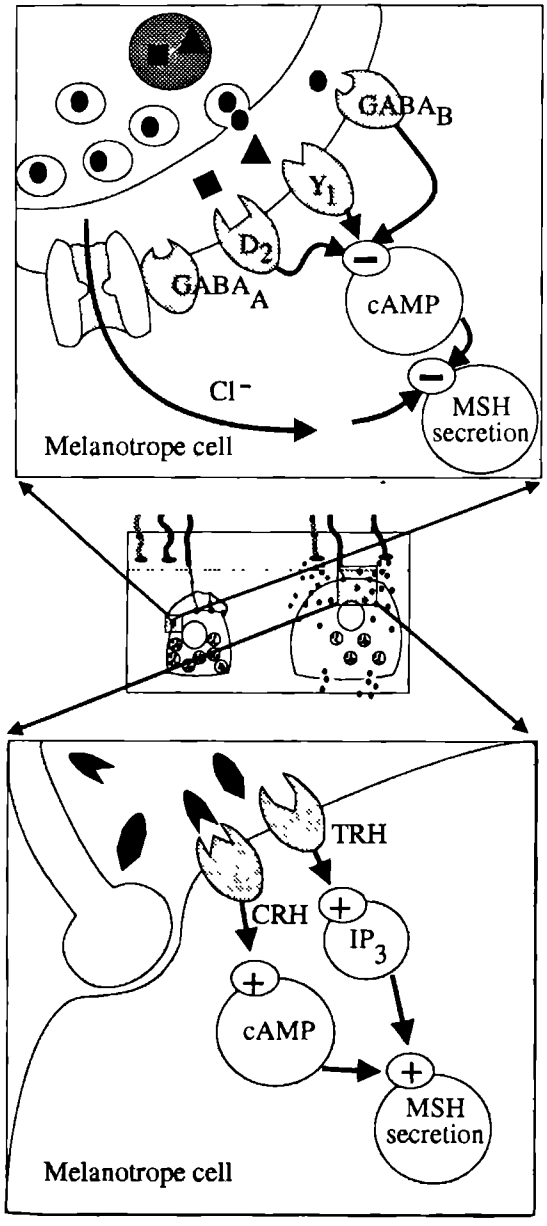


Fig. 5. Detail from figure 3 of the regulation of the melanotrope cell. In the upper box the inhibitory innervation is shown. NPY, dopamine and/or GABA cause via different receptors a reduction of the cAMP concentration or a chloride influx, which in turn are responsible for the decrease of α-MSH secretion. Stimulation of the α-MSH secretion is reached by secretagogues from the pars nervosa that reach the receptors on the melanotropes by diffusion. CRH increases the cAMP concentration and TRH is thought to act via IP_3.

neurons have also been proposed as the inhibitory control centers of amphibian melanotropes, but these claims need to be confirmed.

It is now evident that regulation of the melanotrope cell is more complex than inhibition through dopamine only. Superfusion experiments performed with pars intermedia tissue revealed that inhibition of melanotrope cell activity occurs after administration of neuropeptide Y (NPY; Verburg-Van Kemenade, 1987a), γ-aminobutyric acid (GABA; Verburg-Van Kemenade, 1986a), dopamine (DA; Verburg-Van Kemenade, 1986a,b) and noradrenaline (NA; Bower et al., 1974; Verburg-Van Kemenade, 1986c), whereas stimulation of the release of α-MSH was seen after administration of thyrotropin-releasing hormone (TRH; Verburg-Van Kemenade, 1987b) and corticotropin-releasing hormone (CRH; Verburg-Van Kemenade, 1987c). Immunocytochemical studies at the electron microscopical level have shown that a NPY-, DA- and GABA-positive terminal network is present in the pars intermedia forming synaptic contacts on the melanotrope cells (De Rijk et al., 1990b). All three neurotransmitters coexist in the same synapse (De Rijk et al., 1992), NPY and DA being stored in electron-dense vesicles and GABA in electron-lucent vesicles (De Rijk et al., 1990a; Van Strien et al., 1991). Pharmacological studies revealed the responsible receptor types (Y_1, D_2, $GABA_A$ and $GABA_B$; De Koning et al., 1991; Jenks et al., 1993; Scheenen et al., 1995). The intracellular mechanism utilized by these receptors to regulate the secretory processes is also known in some detail. Stimulation of the $GABA_B$-, Y_1- and D_2-receptors leads via decrease of the intracellular cAMP levels to inhibition of α-MSH secretion, whereas the $GABA_A$-receptor inhibits this release via chloride influx.

In contrast to the direct neural inhibitory innervation, the stimulatory messengers, CRH and TRH are secreted from the pars nervosa and stimulate the melanotropes via either a portal system (Iturriza, 1969) or via diffusion toward the pars intermedia (Weatherhead, 1983; Verburg-Van Kemenade, 1987b,c; Jenks et al., 1993). CRH increases the intracellular cAMP level and TRH the intracellular IP_3 concentration, both peptides causing a higher transcription rate of POMC mRNA (Jenks et al., 1993). Recent studies showed that melanotrope cells *in vitro* display spontaneous Ca^{2+}-oscillations. These oscillations are influenced by the aforementioned neurotransmitters in the same way as they affect α-MSH secretion. Therefore it is assumed that the Ca^{2+}-oscillations are the driving force for α-MSH secretion (Scheenen et al., 1994a,b).

AIM AND OUTLINE OF THE THESIS

As described above, the presumed hypothalamic origin of the melanotrope cell influencing neural messengers in *Xenopus laevis* has been debated over the years. Especially for the location of the inhibitory neurons a number of hypothalamic centers, in the adult as well as in the developing brain, have been proposed. This incomplete knowledge formed the motivation for the present study. From the onset it was felt that the earlier demonstration of coexistence of several neurotransmitters in axon terminals in the pars intermedia might facilitate the search for the cell bodies of these neurons in the hypothalamus. At the start, data on the distribution of the above mentioned neurotransmitters in the brain of *X. laevis* were scarce; some earlier reports were available on the distribution of dopamine in the brain of *Xenopus* tadpoles (Terlou and Ploemacher, 1973; Terlou et al., 1974) and in the brain of *Rana* the distribution of dopamine (Carr et al., 1991), NPY (Andersen et al., 1993; Danger et al., 1985) and GABA (Franzoni and Morino, 1989) had been reported.

The first aim of the studies presented in this thesis is to locate the brain centers that inhibit and stimulate the activity of the melanotrope cells in the pars intermedia of *Xenopus*. Secondly, there is focus on the development and activity of the inhibitory centers of the melanotrope cells in relation to background adaptation. Different techniques have been applied to investigate the distribution of the neural factors, the activity of these immunopositive centers and their connection to the pars intermedia. In **chapter 1**, using immunocytochemistry, a description is given of the distribution of neurons and fibers in the central nervous system that are immunoreactive for antibodies against dopamine and its synthesizing enzyme tyrosine hydroxylase. Various catecholaminergic populations have been detected in the adult *Xenopus* brain. In the diencephalon dopaminergic neurons occur in the anterior preoptic nucleus (POa), the suprachiasmatic nucleus (SC), the posterior thalamic nucleus (P), the posterior tubercle (TP) and the nucleus of the paraventricular organ (NPV).

The development of these dopaminergic neuronal populations has been investigated in different stages of *Xenopus* larves and is dealt with in **chapter 2**. Dopamine is found in early embryonic stages (39-40) in the SC, TP and NPV. Later in development the posterior thalamic nucleus (stage 53) and anterior

preoptic nucleus (stage 59) start to produce dopamine

Terlou et al (1974) proposed that the dopaminergic neurons that are involved in the regulation of background adaptation originate in either the nucleus of the paraventricular organ or the dorsal infundibular nucleus (NID) The dopaminergic nature of these two nuclei was confirmed immunocytochemically Hypothalamic nuclei that beside dopamine also contain NPY- or GABA-positive neurons, are the SC and the TP (NID of Terlou et al , 1974) In **chapter 3**, the SC and TP are proposed to be involved in the regulation of background adaptation

The distribution of NPY-producing cell bodies and fibers in the developing and adult brain of *Xenopus* is described in **chapter 4** In the diencephalon the SC (39-41), the ventromedial (43/44) and posterior thalamic nuclei (43/44), the ventral infundibular nucleus (43/44) and TP (43/44) contain NPY-positive neurons Between brackets the stages are given of the first time these neurons produce NPY The suprachiasmatic nucleus is around stage 39/40 capable of producing both NPY and dopamine The posterior tubercle starts to express dopamine at stage 39/40 but NPY only from stage 43/44 onwards

The activity of NPY-producing diencephalic neurons under different background conditions has been investigated immunocytochemically using anti-bodies against NPY and with *in situ* hybridization using a *Xenopus*-specific preproNPY-mRNA probe The results, presented in **chapter 5**, show an immunoreaction and a positive hybridization signal in neurons of the suprachiasmatic nucleus and the ventromedial thalamic nucleus Quantitative image analysis revealed that the suprachiasmatic neurons were, however, only positive in animals adapted to a white background In white animals an active, NPY-producing and releasing SC seems necessary to inhibit the melanotropes

As a coexistence of NPY and DA has been established in the synapses in the pars intermedia, the relevant regulatory brain centers should also contain NPY and DA Confocal laser scanning microscopy, using different antisera and fluorochromes on the same tissue section, showed different populations of neurons in the suprachiasmatic nucleus, some neurons revealing only NPY or only DA, others containing both NPY and DA These results are discussed in **chapter 6**

Chapter 7 deals with results obtained after application of retrograde tracers, the carbocyanine dyes, DiI and DiO into the pars intermedia and pars

nervosa. Synaptic innervation of the pars intermedia by the suprachiasmatic nucleus and by the locus coeruleus was shown, as neurons in these nuclei became retrogradely labeled. Application of these dyes into the pars nervosa demonstrated that the magnocellular nucleus projects to the pars nervosa. Moreover, optic efferents, visualized with horse radish peroxidase administered to the cut ends of the optic nerve, appeared to terminate in the suprachiasmatic nucleus abutting NPY-immunoreactive neurons.

In **chapter 8**, the main results are discussed in light of the aim of this thesis.

CHAPTER 1

Distribution of tyrosine hydroxylase and dopamine immunoreactivities in the brain of the South African clawed toad *Xenopus laevis*

With Agustin González and Wilhelmus J. A. J. Smeets

in Anatomy and Embryology ***187****; 193-201 (1993)*

ABSTRACT

The distribution of dopamine (DA) and the biosynthetic enzyme tyrosine hydroxylase (TH) has been studied immunohistochemically in the brain of the adult South African clawed toad, *Xenopus laevis* The goals of the present study are, firstly, to provide detailed information on the DA system of the brain of a species which is commonly used in laboratories as an experimental model and, secondly, to enhance our insight into primitive and derived characters of this catecholaminergic system in amphibians DA-immunoreactive cell bodies are present in the olfactory bulb, the preoptic area, the suprachiasmatic nucleus, the nucleus of the paraventricular organ and its accompanying cells, the nucleus of the posterior tubercle, the posterior thalamic nucleus, the midbrain tegmentum, around the solitary tract, in the ependymal layer along the midline of the caudal rhombencephalon, and along the central canal of the spinal cord In contrast to the DA antiserum, the TH antiserum fails to stain the liquor-contacting cells in the paraventricular organ On the contrary, the latter antiserum reveals additional immunoreactive cell bodies in the olfactory bulb, the isthmic region and the caudal brainstem Both antisera yield an almost identical distribution of fibers Distinct fiber plexuses are observed in the olfactory bulb, the basal forebrain, the hypothalamus and the intermediate lobe of the hypophysis Features that *Xenopus* shares with other anurans are the larger number of DA-immunoreactive cells, which are generally smaller in size than those observed in urodeles, and the lack of DA-immunoreactive fibers in pallial structures On the other hand, the paired midbrain DA cell group and the innervation of the tectum of *Xenopus* resemble those found in the newt rather than those in frogs Despite the existence of these species differences, the brain of *Xenopus* offers an excellent model for studying general aspects of neurotransmitter interactions and the development of catecholamine systems in this class of vertebrates

INTRODUCTION

The brain of the South African clawed toad, *Xenopus laevis,* is particularly suitable for the study of developmental aspects of neurotransmitter systems, because of the possibility of hormone-induced breeding, the availability of an accurate timetable of development (Nieuwkoop and Faber,

1967), and the ease of maintenance of this species under laboratory conditions An additional advantage is that neither the developing nor the adult brains of *Xenopus* contain much of the neuromelanin that obscures the immunoreactive cell bodies and fibers in other amphibians Moreover, this species is widely used as an experimental model not only for studying fundamental brain mechanisms such as, for example, background adaptation (De Rijk, 1991) but also for basic processes underlying brain development (*e g* Tay and Straznicky, 1982, Thors et al , 1982, Van Mier and Ten Donkelaar, 1984, Van Mier et al , 1985, 1986, Van der Linden, 1990) Detailed knowledge of the chemoarchitecture of the brain of *Xenopus* may, therefore, be helpful in understanding the functional significance of neurotransmitter systems in both the developing and adult animal Another point to be noted is that recent studies of neuropeptidergic systems in amphibians have revealed considerable differences between species (Gonzalez and Smeets, 1992a,b) For example, the relatively dense vasotocinergic innervation of the brain of *Xenopus laevis* resembles that found in the urodele *Pleurodeles waltlii,* rather than that in the taxonomically more closely related frog, *Rana ridibunda*

The present study has a double purpose First, it will provide detailed information on the dopamine system in the adult *Xenopus* brain which will serve as a basis for future developmental and hodological studies Secondly, by comparing the results obtained in *Xenopus* with those in other amphibians (Gonzalez and Smeets, 1991) insight will be enhanced into primitive and derived characters of the dopamine system in amphibians and, more generally, vertebrates In this regard, *Xenopus* is of particular interest since this species is strictly aquatic and considered a primitive anuran

MATERIALS AND METHODS

Fourteen adult South African clawed toads (8 females, 6 males) were used Anesthesia was achieved by immersion in a 0 3% solution of tricaine methanesulfonate (Sandoz, MS-222) Animals were perfused transcardially with Ringer's solution followed by either a mixture of 4% paraformaldehyde, 0 05% glutaraldehyde and 0 2% picric acid in 0 1 M phosphate buffer (pH 7 4), or of 5% glutaraldehyde in 0 05 M sodium-acetate buffer (pH 4 0) The latter fixation is required for DA-immunohistochemistry but is also suited to the TH

antiserum

Brains were cut on a freezing microtome at 40 µm in frontal (n=8), horizontal (n=3), and sagittal (n=3) planes The further steps in the immunohistochemical procedures are largely the same as previously described for other amphibians (González and Smeets, 1991)

In brief, for DA immunohistochemistry were used

1. Rabbit anti-DA antiserum (generously provided by Dr R M Buijs, Amsterdam), diluted 1.2000 for 16 h.
2 Swine antirabbit antiserum (Nordic), diluted 1 50 for 1 h
3. Rabbit peroxidase anti peroxidase (Dakopatts), diluted 1.800, for 1 h.

For TH immunohistochemistry were used:

1 Mouse anti-TH antiserum (Incstar), diluted 1 1000, for 48-60 h
2. Goat antimouse antiserum (Nordic), diluted 1 100, for 3 h
3 Rat peroxidase antiperoxidase antiserum (Incstar), diluted 1:800, for 2 h

For details about the specificity of the antibodies, the reader is referred to the previous paper. It should be noted, however, that in the present study, the TH antiserum was purchased from Incstar instead of Eugene Tech. This replacement did not result in a different pattern of immunoreactivity, as checked in brain sections of *R. ridibunda* and *P. waltlii*, but it yielded generally a stronger staining of cell processes

Evaluation and presentation of the results

The distribution of the DA- and TH-immunoreactive cell bodies and fibers in the brain of *Xenopus laevis* is charted in representative transverse sections of which the levels are indicated in figure 1. To facilitate a comparison of the results obtained with the immunohistochemical procedures, at each level TH-immunoreactivity is depicted on the left side and DA- immunoreactivity on the right side (Fig. 2)

Drawings were made by means of a camera lucida, and sections counterstained with cresyl violet facilitated the interpretation of the location of immunoreactive elements The nomenclature in the present study is that of González and Smeets (1991, 1992b).

The DA and TH antibodies used in the present study reveal patterns of immunostaining that are the same for all animals studied. As can be expected on the basis of the catecholamine biosynthetic pathway, more cell bodies are immuno-positive to TH than to DA antibodies. This suggests that these cells are either noradrenergic or adrenergic (González, 1992). Comparison of adjacent sections leads to the conclusion that DA-immunoreactive perikarya are also TH-immunoreactive. One obvious exception is the nucleus of the paraventricular organ, where cells are immunopositive for DA but immunonegative for TH antibodies. Moreover, cell bodies stain generally better with the TH antiserum, whereas DA antiserum is superior in fiber staining.

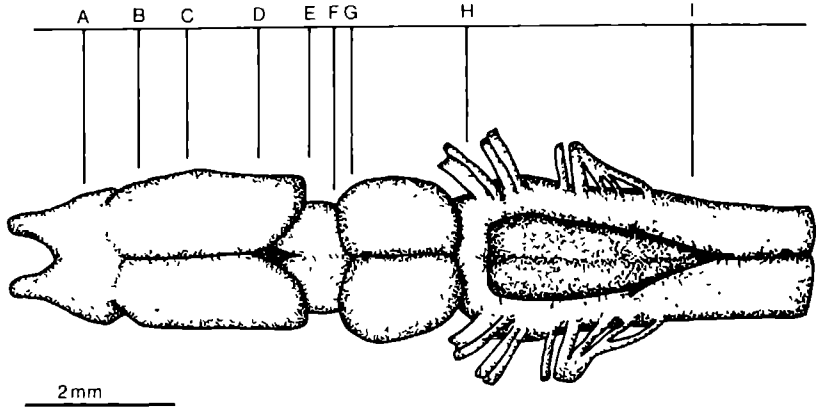
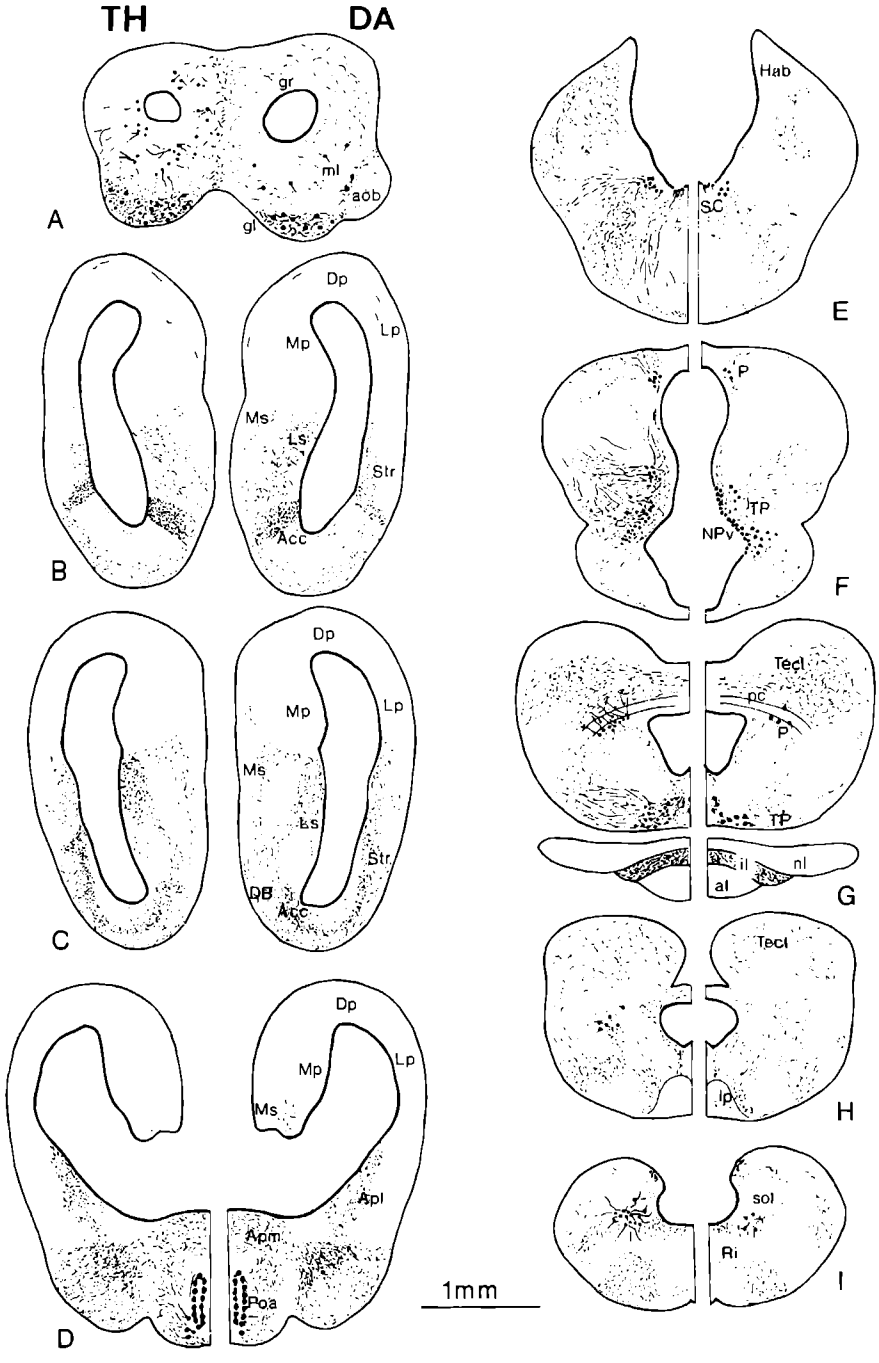


Fig. 1. Dorsal view of the brain of the South African clawed toad *Xenopus laevis* The letters on top refer to those of the transverse sections of **figure 2**

Distribution of DA- and TH-immunoreactive cell bodies

The most rostral DA- and TH-immunoreactive cell bodies in *Xenopus* are located in the olfactory bulb around the glomeruli, and in both the external plexiform layer and the mitral cell layer (Fig. 2A). The cells in the glomerular layer are considerably larger than those in the latter two layers (Fig. 3). Rostrally, where the two bulbs are fused, cell bodies are present in the midline and some of their processes cross to the contralateral side (Fig. 4). Only with the TH antiserum additional cell bodies are found in the internal granular layer. No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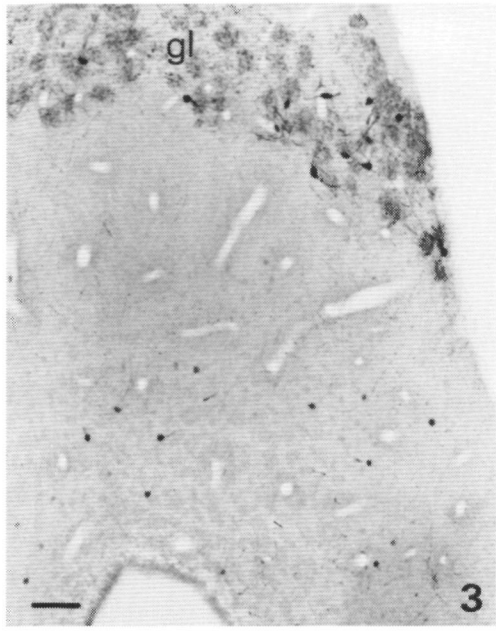
DA- or TH-immunoreactive cell bodies are found in the telencephalon proper (Figs 2B-D)

The diencephalon harbors the majority of DA- and TH-immunoreactive cell bodies Cells are found in the anterior preoptic area, the suprachiasmatic nucleus, the nucleus of the paraventricular organ and its accompanying cells, the nucleus of the posterior tubercle, and the posterior thalamic nucleus (Figs 2D-G, 5-7) On the basis of their morphology, the DA- and TH-immunoreactive cell bodies in the diencephalon can be subdivided into two categories One category comprises cell bodies that are in direct contact with the cerebrospinal fluid (CSF) of the third ventricle by means of short processes with club-like endings The other category is constituted by cells that do not contact the ventricle Most of the DA- and TH-immunoreactive cells in the anterior preoptic area and the DA-immunoreactive cells in the paraventricular organ (Fig 6) are CSF-contacting The cell bodies in the remaining cell groups belong to the second category, although some cells of the suprachiasmatic nucleus may be liquor-contacting (Fig 5)

In the brainstem, DA- and TH-immunoreactive cell bodies are present in the medial part of the midbrain tegmentum, around the solitary tract, and in the area postrema at the obex level (Figs 2G-I,7,8) The cells in the midbrain tegmentum constitute separate groups on both sides of the midline The majority of the cells extend their dendrites mediolaterally, although a few cell bodies lying in the midline have processes that are directed mainly dorsoventrally (Figs 2G, 7) At the caudal rhombencephalic levels, DA- and TH-immunoreactive cell bodies lie around the solitary tract (Fig 2I) The cells are large and multipolar and are mainly located medial and ventral to the tract At the level of the obex, the cells of both sides fuse above the ventricle, in the area postrema (Fig 8) Additional

Fig. 2A-I. Diagrams of transverse sections through the brain of *Xenopus laevis* at the levels indicated in **figure 1**, showing the position of TH- and DA-immunoreactive cell bodies (*large dots*) and fibers (*small dots, wavy lines*) *Acc*, nucleus accumbens, *aob*, accessory olfactory bulb, *Apl*, lateral amygdala, *Amp*, medial amygdala, *DB*, diagonal band of Broca, *gl*, glomerular layer, *gr*, granule cell layer, *Hab*, habenula, *Ip*, interpeduncular nucleus, *Lp*, lateral pallium, *Ls*, lateral septum, *ml*, mitral cell layer, *Mp*, medial pallium, *Ms*, medial septum, *NPv*, nucleus of the paraventricular organ, *P*, posterior thalamic nucleus, *pc*, posterior commissure, *PD*, hypohysis, pars distalis, *PI*, hypophysis, pars intermedia, *PN*, hypophysis, pars nervosa, *Poa*, anterior preoptic nucleus, *Ri*, inferior reticular nucleus, *SC*, suprachiasmatic nucleus, *sol*, solitary tract, *Str*, striatum, *Tect*, tectum mesencephali, *TP*, posterior tubercle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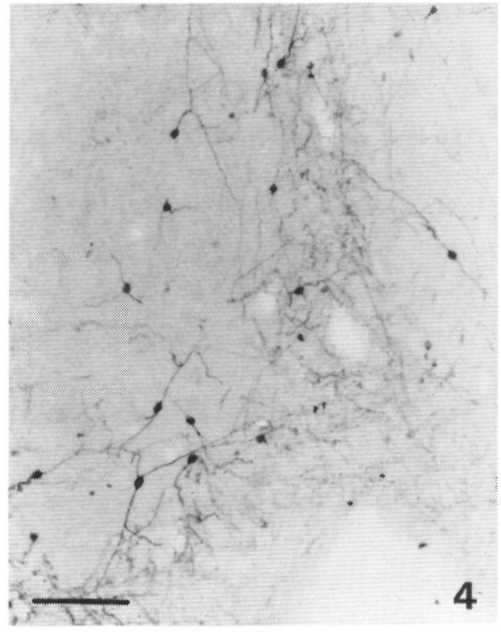
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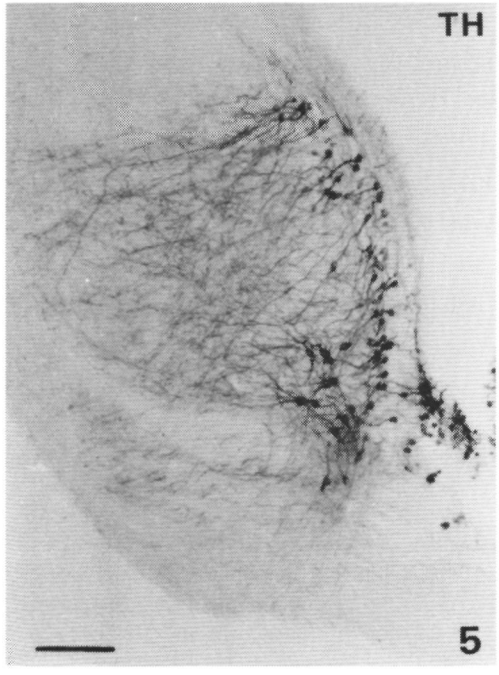
TH
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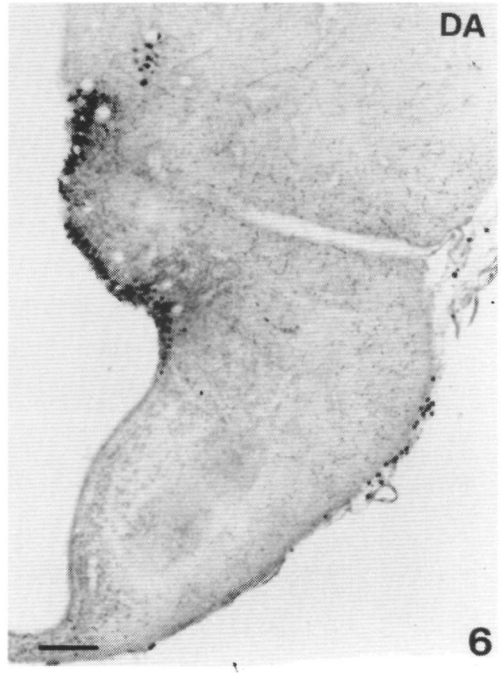

DA
6

cell bodies occur in the ependymal layer along the midline of the caudal rhombencephalon and along the central canal of the spinal cord where they contact the CSF.

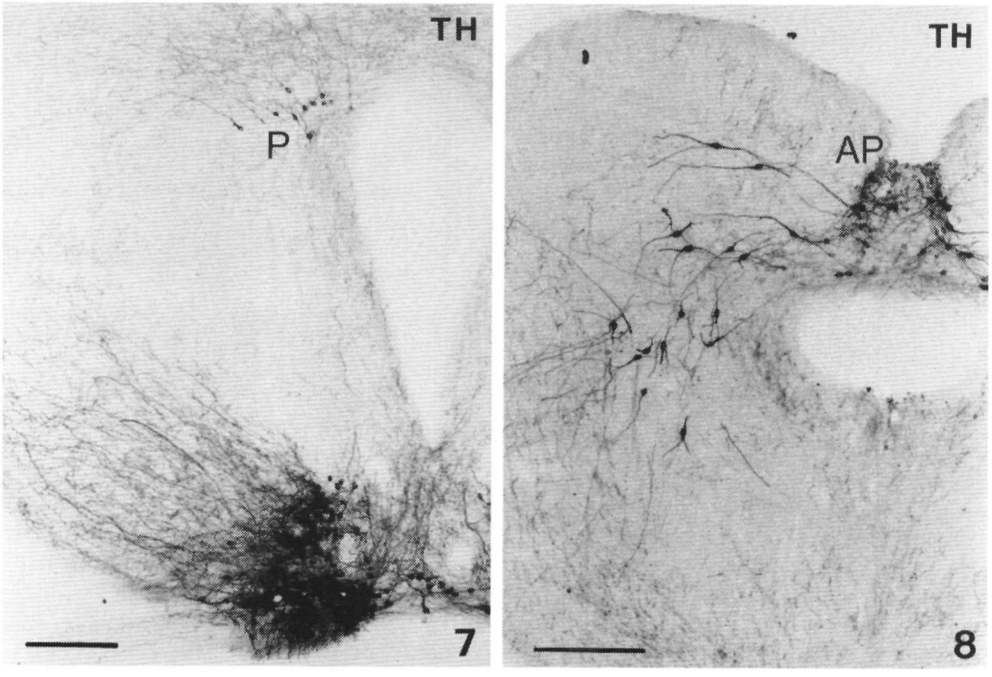
Distribution of DA- and TH-immunoreactive fibers

The two antisera reveal an almost identical distribution of fibers. Distinct plexuses of immunoreactive fibers are observed in the glomerular layer of the olfactory bulb, and in the nucleus accumbens, the striatum, the dorsal portion of the lateral septum, and the amygdala of the telencephalon proper (Figs 2A-D). The olfactory bulb contains many immunoreactive fibers in the glomerular layer. In the external plexiform and the mitral cell layers, a considerable number of immunoreactive fibers, probably dendrites, are present (Figs 3,4). Only a few fibers are observed in the internal granular layer. In the telencephalon proper, the DA- and TH-immunoreactive fibers are almost exclusively confined to the subcortical areas (Figs 2B-D). The most obvious plexus of immunoreactive fibers occurs in the nucleus accumbens. At very rostral levels, a single plexus is visible, but more caudally, this plexus is subdivided into ventromedial and ventrolateral portions separated by a region that is poor in immunoreactive fibers (Figs 2B,9). Another distinct plexus of DA- and TH-immunoreactive fibers is found in the striatum (Figs 2C,10). The immunoreactive fibers course primarily in the intermediate zone, probably contacting the proximal parts of the dendrites of the striatal neurons. In the lateral septum, the DA- and TH-immunoreactive fibers are inhomogeneously distributed and occur predominantly in its dorsolateral part (Figs 2B,C). The plexus of the amygdaloid complex is rostrally continuous with that of the striatum (Fig. 2D).

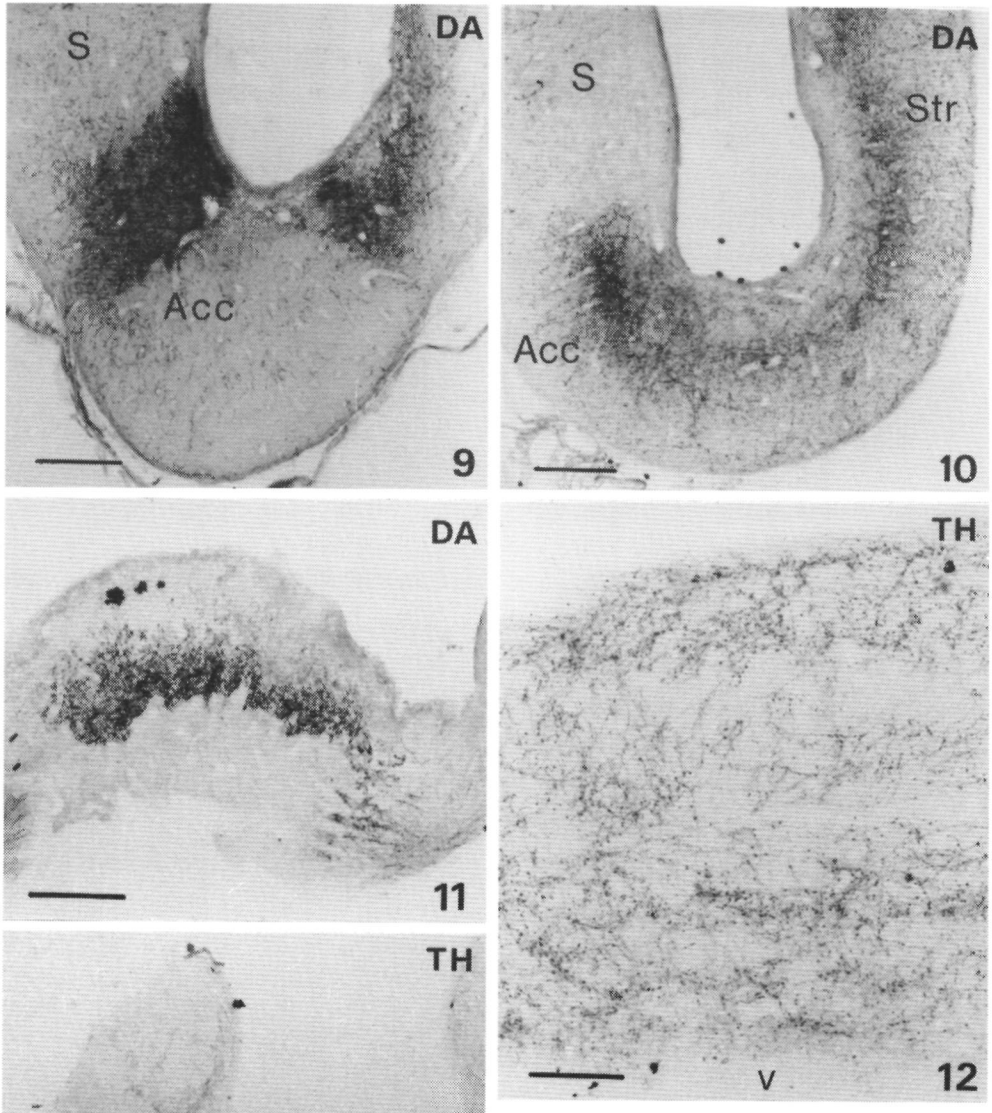
Figs 3-6 Photomicrographs from horizontal (**Fig. 3**) and transverse (**Figs 4-6**) sections through the brain of *Xenopus* showing the DA- and TH-immunoreactive cell bodies and fibers in the forebrain Note in **figure 3** the rather large periglomerular DA-immunoreactive cell bodies and the high immunoreactivity in the glomerular layer (gl) **Fig. 4** clearly illustrates the morphology of the, compared to the periglomerular cells, rather small immunoreactive cells along the line of fusion between the two olfactory bulbs **Fig. 5** shows the TH-immunoreactive cell bodies in the suprachiasmatic nucleus, whereas **figure 6** illustrates the DA-immunoreactive cells in the nucleus of the paraventricular organ and its accompanying cells Scalebar = 100 μm

Numerous DA- and TH-immunoreactive fibers cross the midline in the anterior commissure and interconnect the amygdalar immunoreactive plexuses. A distinct plexus is also found in an area that encompasses the lateral forebrain bundle (Fig. 2D).

At rostral diencephalic levels, a rather dense plexus of immunoreactive fibers is observed in the lateral preoptic area, which is primarily formed by the coarse fibers of immunoreactive cell bodies in the suprachiasmatic nucleus (Figs 2D,E). Other areas in the diencephalon that contain a moderately dense plexus of DA- and TH-immunoreactive fibers are the ventral habenular nucleus, the anterior and central thalamic nuclei and the hypothalamus (Figs 2E,F). Numerous immunoreactive fibers are present in the lateral hypothalamus coursing toward the median eminence, where they distribute to the intermediate lobe of the hypophysis (Figs 2F,G,11).



Figs 7 and 8. Photomicrographs of transverse sections showing the TH-immunoreactive cell bodies in the posterior thalamic nucleus (P) and the midbrain tegmentum (**Fig. 7**), and around the solitary tract and in the area postrema (AP; **Fig. 8**). Scalebar = 100 μm.



Figs 9-13. Photomicrographs of transverse sections of *Xenopus* showing the DA- and TH-immunoreactive fibers in the nucleus accumbens (**Fig. 9**), the striatum (**Fig. 10**), the intermediate lobe of the hypophysis (**Fig. 11**), the midbrain tectum (**Fig. 12**), and the rhombencephalic tegmentum (**Fig. 13**). *Acc*, nucleus accumbens; *S*, septum; *Str*, striatum; *v*, ventricle. Scalebar = 100 μm.

The two antisera reveal an almost identical staining pattern of fibers in the brainstem A rather dense network of immunoreactive fibers is found in the midbrain tectum (Fig 12) Although all tectal layers contain DA- and TH-immunoreactive fibers, their density in the superficial and deep tectal zones is higher than that in the intermediate zone Ventrally, in the midbrain tegmentum, moderately dense plexuses of immunoreactive fibers are present in the paraventricular gray, the raphe and ventrolateral tegmentum These fibers can be traced caudally, in a similar position, to cervical spinal cord levels (Figs 2H,I,13) In the dorsal alar plate of the rhombencephalon numerous DA- and TH-immunoreactive fibers are found in the lateral line area (Fig 13)

DISCUSSION

In this study the distribution of the catecholamine dopamine and its synthetic enzyme tyrosine hydroxylase is immunohistochemically studied in the brain of the South African clawed toad, *Xenopus laevis* Both the DA and TH antibodies yielded a consistent staining pattern of cell somata and fibers, thus underscoring once again the usefullness of these antisera Here we consider first the distribution of DA- and TH-immunoreactive neuronal structures in *Xenopus* in relation to the results obtained in other amphibians Special attention is paid to species differences that contribute to a better understanding of the evolution of the catecholamine systems in the brain of vertebrates Secondly, we show that *Xenopus* offers a particularly suitable model to study developmental and basic aspects of catecholamine systems in amphibians

Comparison with previous studies

The distribution of DA- and TH-immunoreactive cell bodies and fibers of *Xenopus* generally resembles the pattern observed in *R ridibunda* and *P waltlii* (González and Smeets, 1991) As expected, the TH antiserum stains more neuronal structures than the DA antiserum Preliminary observations (González, 1992, Gonzalez and Smeets, 1993) indicate that some of these cells are noradrenergic or adrenergic, whereas others, for example the TH-immunoreactive cells in the internal granular layer of the olfactory bulb, probably contain DOPA as endproduct of the biosynthesis of catecholamines

(González and Smeets, 1991). However, although the catecholaminergic nature of the cells in the paraventricular organ of *Xenopus* has been demonstrated, by means of the formaldehyde-induced fluorescence technique (Terlou and Ploemacher, 1973) and DA immunocytochemistry (present study), the TH antiserum fails to stain the CSF-contacting cells in this organ. This finding is in agreement with previous results obtained for other amphibians (Smeets and González, 1990; González and Smeets, 1991) and supports the hypothesis that these cells, which have only been observed in non-mammalian vertebrates, accumulate rather than synthesize dopamine (Nakai et al., 1977; Smeets and González, 1991; Smeets and Steinbusch, 1990; Smeets et al., 1991).

In a previous study (González and Smeets, 1991), comparison of the dopamine system of the anuran *R. ridibunda* with that of the urodele, *P. waltlii*, revealed five major differences:

1. The DA- and TH-immunoreactive cells in the newt are smaller in number but larger in size than those in the brain of the frog.

2. The cells in the midbrain of *P. waltlii* do not, as in *Rana*, form a single group, but constitute separate groups on both sides of the midline. The processes of the cells in the midbrain of the former species have a predominantly mediolateral orientation, whereas those of the latter species course mainly ventrodorsally.

3. Whereas in *Rana* immunoreactive fibers are absent in cortical areas, in *Pleurodeles* more or less developed plexuses of DA- and TH-immunoreactive fibers are present in all pallial areas.

4. In *Pleurodeles* the striatum and not the nucleus accumbens, as in *Rana*, contains the most densely stained DA- and TH-immunoreactive plexus of the basal forebrain.

5. In addition the DA- and TH-immunoreactive fibers in the deep plexiform tectal layers observed in both the frog and the newt, additional fibers are present in the superficial tectal layers.

A comparison of the dopamine system of *Xenopus* with that of the two other amphibian species, reveals that *Xenopus* shares some features with *Rana* and others with *Pleurodeles*. The dopamine systems of both anuran species resemble each other in having a larger number of immunoreactive cells, which are generally smaller in size than those of *Pleurodeles*. Moreover, in *Rana* and

Xenopus pallial structures are devoid of immunoreactivity, whereas in the newt the corresponding regions contain numerous immunoreactive fibers. On the other hand, the rather dense plexuses of DA- and TH-immunoreactive fibers in both the deep and superficial tectal layers in *Xenopus* resemble the pattern observed in *P. waltlii* rather than that in *R. ridibunda*. From a phylogenetic point of view, the presence in *Xenopus* of a paired midbrain dopaminergic cell group and an almost equally dense innervation of the nucleus accumbens and the striatum is of particular interest. On the basis of previous observations in amphibians, it has been suggested that the mesolimbic system is particularly well developed in frogs, whereas the mesostriatal system prevails in the newt (González and Smeets, 1991). However, the present findings in *Xenopus* do not unequivocally support this notion. The midbrain dopaminergic cell group of *Xenopus* resembles more the corresponding group in the newt than that in the frog, especially with respect to the location of the cell bodies and the mediolateral orientation of their processes. Moreover, whereas in *R. ridibunda* the nucleus accumbens, and in *P. waltlii* the striatum contains the most dense DA-/TH-immunoreactive plexus, the two structures are almost equally densely innervated in the clawed frog. Therefore, a paired cell group in the midbrain tegmentum is not necessarily correlated to the mesostriatal system as is obvious from the very dense immunoreactive plexus in the nucleus accumbens of *Xenopus*. The presence of a paired group of cells with mediolaterally oriented processes in amphibians (*Xenopus*, *Pleurodeles*), cartilaginous fishes (Meredith and Smeets, 1987; Northcutt et al., 1988; Stuesse et al., 1990, 1991; Stuesse and Cruce, 1992), reptiles (Smeets et al., 1986, 1987; Brauth, 1988; Smeets, 1988) and mammals (*e.g.*, Haber and Groenewegen, 1989) suggests that this is the primitive condition. The unpaired, midline group in *Rana* should, therefore be considered as a derived condition. Another remarkable feature that *Xenopus* shares with *Pleurodeles* is that DA- and TH-immunoreactive fibers probably contact the proximal dendrites of the striatal cells, whereas in *Rana* the majority of the immunoreactive fibers lie in the cellular layer. The general impression is, therefore, that the basal forebrain organization of *Xenopus* has more in common with that of the newt than with that of *Rana*. Currently, studies combining immunohistochemical and tract tracing techniques are in progress to find possible fundamental differences in mesostriatal connections.

From the foregoing it is clear that *Xenopus* may serve as an excellent model to study developmental and general aspects of catecholamine systems in amphibians. In the adult brains of several amphibian species, however, differences are observed that are important to note, since they may contribute to a better understanding of the evolution of these systems. In this respect, the development of the midbrain cell group and the connections between the midbrain and the basal forebrain deserve special attention.

The present study also forms part of a project that aims to determine which structures in the brain of *Xenopus* are involved in the hypophyseal control of background adaptation (see *e.g.* Roubos, 1992). It has been demonstrated that dopamine co-exists with GABA and neuropeptide Y (NPY) in axon varicosities that innervate the intermediate lobe of the hypophysis (De Rijk, 1991; De Rijk et al., 1992). Similar observations have been made in *R. ridibunda* (Tonon et al., 1992), suggesting that we are dealing here with a basic feature of brain organization. Preliminary studies of *Xenopus* by means of TH and NPY immunohistochemistry on adjacent sections revealed that cell bodies in the suprachiasmatic nucleus are immunoreactive for both antisera, making this nucleus a likely candidate for controlling the melanotrope cells in the pars intermedia of the hypophysis (Tuinhof et al., 1992).

ACKNOWLEDGEMENTS

The authors are much indebted to Dr E.W. Roubos for critically reading the manuscript, Mrs B. Jorritsma-Byham and Miss P.G.P. Wismans for technical assistance, and Mr D. de Jong for preparing the photomicrographs. The study was financially supported by NATO Colloborative Research Grant CRG 910970.

CHAPTER 2

Ontogeny of catecholamine systems in the CNS of anuran amphibians: an immunohistochemical study with antibodies against tyrosine hydroxylase and dopamine

With Agustín González, Oscar Marín and Wilhelmus J. A. J. Smeets

in Journal of Comparative Neurology ***346;*** *63-79 (1994)*

ABSTRACT

To get more insight into developmental aspects of catecholamine systems in vertebrates, in particular anuran amphibians, these systems were studied immunohistochemically in embryos and larvae of *Xenopus laevis* and *Rana ridibunda* Antisera against tyrosine hydroxylase (TH) and dopamine (DA) revealed that catecholamine systems are already present at early embryonic stages The first dopamine group to be detected was found ventral to the central canal of the spinal cord of *Xenopus*, soon followed by DA cell groups in the posterior tubercle, the hypothalamic paraventricular organ, the accompanying cell group of the paraventricular organ and the suprachiasmatic nucleus Although weakly TH-immunoreactive cells were found in the olfactory bulb at about the same embryonic stages, DA-immunoreactivity was not detected until premetamorphic stage 49 Dopamine cell groups in the caudal brainstem, midbrain and pretectum appeared at late premetamorphic stages, whereas the preoptic group was first observed at the metamorphic climax stage

Rana showed an almost similar timetable of development of catecholamine cell groups, except for the caudal brainstem group, which was already present at the end of the embryonic period When compared with previous studies by means of formaldehyde-induced fluorescence technique, it becomes clear that TH/DA immunohistochemistry enables an earlier detection of catecholamine cell groups and fiber systems in anuran amphibians The present study also revealed that the DA-immunoreactive cells of the hypothalamic paraventricular organ never stained with the TH antiserum during development, thus supporting their putatively DA accumulating nature Another notable result is the site of origin and rather late appearance of the midbrain dopaminergic cell group It is suggested that the latter cell group only partly corresponds to the ventral tegmental area and substantia nigra of amniotes

INTRODUCTION

The anurans constitute the order of amphibians that includes frogs and toads These animals are known to undergo striking changes during development Changes occur not only during the embryonic period, but also during postembryonic development, generally referred to as metamorphosis During

metamorphosis a larva, which is adapted to a specific environment, usually transforms into a young adult with more or less different needs imposed on it by its specific environment (Wald, 1981; Fritzsch, 1990). Whereas the remarkable, external changes during the transformation from free-living tadpoles into froglets have been extensively analyzed and described, current knowledge of metamorphic changes in the central nervous system (CNS) is limited.

A few studies by means of formaldehyde-induced fluorescence technique have dealt with developmental aspects of the catecholamine systems of amphibians (Bartels, 1971; Terlou and Ploemacher, 1973; Notenboom, 1974; McKenna and Rosenbluth, 1975; Sims, 1977; Corio and Doerr-Schott, 1988). These studies focussed primarily on hypothalamo-hypophyseal relationships and provide little information about the ontogeny of catecholamine cell groups and fibers. Morever, these studies dealt, in general, only with late larval stages.

The main purpose of the present study is, therefore, to get a better insight into the development of catecholamine systems in representatives of a class of vertebrates which marks a crucial point in evolution, *i.e.*, the transition from an aquatic to a terrestrial life style. A better understanding of the development of these systems in amphibians may provide the answers to several fundamental questions, such as the origin of the midbrain dopaminergic cell groups of anamniotes and the nature of the dopamine-containing cerebrospinal fluid (CSF)-contacting cells of the hypothalamic paraventricular organ. With this in mind we have studied the appearance and development of catecholamine cell groups and fiber systems in the brains of two anuran species, *Xenopus laevis* and *Rana ridibunda*. Antisera against the enzyme tyrosine hydroxylase (TH) have been applied to reveal the overall distribution of catecholaminergic neuronal elements in the brain, whereas a dopamine (DA) antiserum has been used to stain selectively dopamine-containing cell bodies and fibers. Consequentely, structures that are immunopositive for TH, but immunonegative for DA likely contain noradrenaline or adrenaline (Smeets and Steinbusch, 1990). TH- and DA antisera used in the present study have been successfully applied before on adult brains of both species (González and Smeets, 1991,1993; González et al., 1993).

The South African clawed toad *Xenopus laevis*, has been selected as core species, because the possibility of hormone-induced breeding, the availability of an accurate timetable of development (Nieuwkoop and Faber, 1967), and the

ease of maintenance of this species under laboratory conditions. An additional advantage is that neither the developing nor the adult brains of *Xenopus* contain much of the neuromelanin that obscures the immunoreactive cell bodies and fibers in other amphibians. The data obtained in *Rana ridibunda*, an anuran that possesses a brain with widely distributed neuromelanin during development (Mensah and Finger, 1975), are primarily used to assess the general features as well as the species differences in the ontogeny of anuran catecholamine systems.

MATERIAL AND METHODS

About sixty *Xenopus laevis* tadpoles, ranging from stage 37 to stage 65, and 35 *Rana ridibunda* tadpoles, ranging from stage 30 to stage 50, were used. Staging was done according to Nieuwkoop and Faber (1967) for *Xenopus*, and Manelli and Margaritora (1961) for *Rana*. To compare with other frogs, the staging by Taylor and Kollros (1946) for *Rana pipiens* or the rather rough staging by Gona et al. (1982) for *Rana catesbeiana* have also been used as references.

Xenopus larvae were obtained by Pregnyl-induced (Organon) breeding and kept in tap water at 20-25°C. The tadpoles of *Rana* were bred from commercially purchased adult frogs and maintained at 20°C throughout their development. The larvae were raised on nettle powder or canned spinach, whereas older larvae and young froglets were fed Tubifex. At appropiate times, embryos and tadpoles were anaesthetized in a 0.3% solution of tricaine methanesulphonate (MS 222; Sandoz) and, subsequentely processed for TH- or DA-immunocytochemistry.

TH-immunocytochemistry

Under anaesthesia, the animals were perfused transcardially with saline followed by a mixture of 4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. Next, the brains and eyes were removed and further fixed in the same mixture for 1-2 hours at room temperature. At early developmental stages, when perfusion was technically impossible, the whole animal was fixed and processed.

After fixation, the brains and eyes were immersed in a solution of 30%

sucrose with 1% $Na_2S_2O_5$ in 0.1 M phosphate buffer (pH 7.1) for 3-5 hours at 4°C, embedded in a solution of 15% gelatin with 30% sucrose and stored for 5-7 hours in a 4% formaldehyde solution at room temperature. The gelatin blocks were cut on a freezing microtome at 40 µm thickness in the transverse and sagittal plane. The sections were collected in phosphate buffer and rinsed three times for 15 minutes in Tris-buffered saline (TBS). The sections were subsequentely processed immunohistochemically according to the peroxidase antiperoxidase (PAP) technique (Sternberger, 1979), using a rabbit anti-TH serum (Eugene Tech) or a mouse anti-TH serum (Incstar). This procedure includes the following steps:

1) incubation with TH antiserum (Eugene Tech, diluted 1:2000 for 12-16 hours; Incstar, diluted 1:1000 for 48-72 hours);

2) incubation with swine antirabbit (diluted 1:50) or goat antimouse antiserum (diluted 1:100) for 3 hours;

3) incubation with rabbit PAP complex (diluted 1:800) or mouse PAP complex (1:500) for two hours; and

4) staining in 0.5 mg/ml 3,3'-diaminobenzidine (DAB, Sigma) with 0.01% H_2O_2 in TBS for 10-20 minutes. In most cases, visualization was enhanced by adding 3.8% of a solution of 1% ammonium sulfate to the DAB-H_2O_2 mixture. Between all steps the sections were rinsed systematically three times for ten minutes in TBS (pH 7.6). Finally, the sections were mounted on glass slides (mounting medium: 0.25% gelatin in Tris-buffer) and, after drying overnight, coverslipped.

DA-immunohistochemistry

Under anaesthesia the animals were perfused transcardially with saline followed by a mixture of 5% glutaraldehyde in 0.05 M sodium-cacodylate and 1% $Na_2S_2O_5$ (pH 7.1). The brains and eyes were removed and further fixed in the same solution for 1 to 2 hours at room temperature. Embedding in gelatine and cutting was carried out as described above. The sections were collected in a Tris-NaCl buffer containing 1% $Na_2S_2O_5$ (pH 7.1).

For DA immunohistochemistry were used:

1) rabbit anti-DA antiserum (generously provided by Dr R.M Buijs, Amsterdam), diluted 1:2000 in TBS containing 0.5% Triton X-100 (TBS-TX, pH 7.6) and 1% $Na_2S_2O_5$ for 16 hours at 4°C;

2) swine antirabbit antiserum (Nordic), diluted 1:50 for one hour at room temperature; and
3) rabbit peroxidase antiperoxidase antiserum (Dakopatts), diluted 1:800 for one hour at room temperature. Further processing of the sections including DAB staining, mounting and coverslipping was the same as used for TH immunohistochemistry.

For details about the specificity of the antibodies, the reader is referred to our previous papers (González and Smeets, 1991; González et al., 1993). The nomenclature in the present study is that of González and Smeets (1991,1993).

RESULTS

The distribution of TH- and DA-immunoreactive cell bodies and fibers has been investigated in animals whose ages varied from late embryonic up to juvenile stages. Since our observations involve tadpoles of two different anuran species (*Xenopus laevis, Rana ridibunda*), some notes on the development of these species are in order. In figure 1, a comparative staging of the development of *Xenopus* and *Rana* is presented. The end of the embryonic period extends over a rather long period of time beginning with the appearance of an operculum that covers the external gills and ending with the total resorption of the external gills.

The larval period, marked by independent feeding, is generally subdivided into three sets of stages (Gona et al., 1982):
1) premetamorphic stages, in which the tadpole merely grows in size and the buds of the hindlimbs appear on the lateral side of the body,
2) prometamorphic stages, characterized by the progressive formation of the hindlimbs. This period ends when the length of the tail is at its maximum and the more drastic changes of the metamorphosis start, and
3) metamorphic climax, marking the period in which the transformation of the tailed larval form into the tailless, four legged juvenile occurs.

In the following sections, we describe the development of catecholamine systems in anurans, keeping these generalized sets of stages in mind. A detailed description of the catecholamine systems in the developing brain of *Xenopus* will be presented first, followed by a brief account of the major events during development in the brain of *Rana*.

The antibodies against TH and DA used in the present study revealed a pattern of immunostaining in the brain that was generally consistent among animals of the same stage. However, in some cases, variation in the intensity of immunostaining was observed among animals treated identically. This variation might be due either to variations in internal development between individuals that are staged exclusively on the basis of external morphological features or to small variations in the technical procedures.

Late embryonic stages

The earliest tadpoles of *Xenopus* that were analyzed correspond to developmental stages 38 to 42. All of them were individuals close to hatching and independent feeding. At these late embryonic stages, rather well developed catecholamine cell groups are present (Fig. 2).

Already in tadpoles of stage 38, distinct TH- and DA-immunoreactive cell bodies are found in the spinal cord, ventral to the central canal (Table 1). These cells extend throughout the length of the spinal cord but do not reach caudal rhombencephalic levels at this early stage. At the caudal spinal cord levels, the number of cells is larger and the cells are more packed together. The TH- and DA-immunoreactive cells in the spinal cord are characterized by a short, thick process that protrudes into the central canal and a thin process that courses laterorostrally (Figs 2,3).

At stage 39, a group of large, TH- and DA-immunoreactive cell bodies appears at caudal diencephalic levels, *i.e.*, in the posterior tubercle (TP) dorsal to the infundibulum (Fig. 4). Slightly later, at stage 40/41, three other groups of catecholamine-containing cells are recognized in the diencephalon, namely the nucleus of the paraventricular organ, its accompanying cells and the suprachiasmatic nucleus (Fig. 2). A remarkable feature of the DA-immunoreactive, liquor (CSF)-contacting cells of the paraventricular organ (Table 1, Fig. 6) is that they never show immunoreactivity with TH antibodies. The accompanying cells of the paraventricular organ, on the contrary, do not contact the CSF but are immunopositive for TH and DA antibodies (Fig. 2F).

The TH- and DA-immunoreactive cell bodies of the suprachiasmatic nucleus constitute a small population of cells dorsal and rostral to the chiasmatic ridge (Fig. 2E). Although these cells lie very close to the wall of the preoptic recess, a distinct relation with the ependymal layer cannot be recognized.

At about the same time, two new groups of TH-immunoreactive cells appear outside the hypothalamus. One group of weakly immunoreactive cells lies in the immature olfactory bulb (stage 42); the other group, also containing weakly stained cells, is found in the isthmic region at the level of the developing cerebellum (stage 41, Figs 2K,5). Some cells of the latter group lie close to the ventricle, whereas others are located at the border between the cell zone and the fiber zone. Weakly stained processes of the TH-immunoreactive cells extend ventrolaterally into the fiber zone. In the case of one tadpole at stage 41, but not others at this stage, a few TH-immunoreactive cell bodies were found in the dorsal part of the diencephalon, close to the midline (Fig. 2H). However, in subsequent developmental stages, neither TH- nor DA-immunoreactive cell bodies were observed in a corresponding position.

At stage 41, several fiber systems are observed that are immunoreactive with TH and DA antisera. TH- and DA-immunoreactive fibers can already be traced to the hypophysis, the basal forebrain, the ventral and ventrolateral parts of the midbrain and the rhombencephalon, and to the marginal zone of the spinal cord (Figs 2,5). At stages 40/41, some fibers leave the main stream fibers in the ventrolateral rhombencephalic tegmentum. They course to the octavolateral area, where they terminate primarily in the area that receives lateral line input (Fig. 2L).

Several features of the distribution pattern of immunoreactivity in larvae just before premetamorphosis (stages 43-45) deserve comments. First, the number of TH- and DA-immunoreactive cell bodies in the posterior tubercle has dramatically increased, particularly in its mediocaudal part. A second feature to be mentioned is the appearance of TH- and DA-immunoreactive cell bodies in

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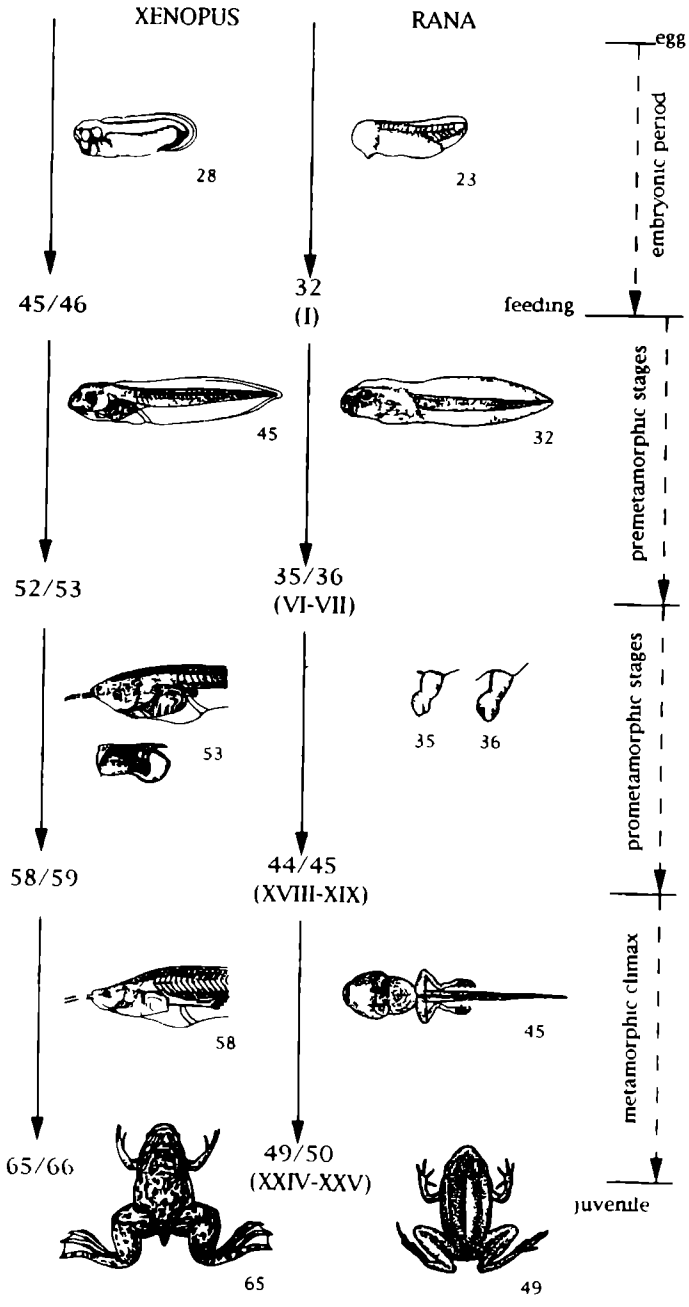
Fig. 1. Developmental staging of *Xenopus* and *Rana* from egg to juvenile The periods of development, as indicated in the **right column**, have been adapted for *Xenopus* (**left column**) after Nieuwkoop and Faber (1967), for *Rana esculenta* (Arabic numbers in **central column**) after Manelli and Margaritora (1961), and for *Rana pipiens* (Roman numbers in central column) after Taylor and Kollros (1946).


XENOPUS
RANA
egg
embryonic period
28
23
45/46
32
(I)
feeding
premetamorphic stages
45
32
52/53
35/36
(VI-VII)
prometamorphic stages
53
35
36
58/59
44/45
(XVIII-XIX)
metamorphic climax
58
45
65/66
49/50
(XXIV-XXV)
juvenile
65
49

the retina (Fig. 6). The cells, which are few in number, lie in the innermost cell row of the inner nuclear layer. They have processes which arborize profusely within the inner layer but do not constitute distinct laminae.

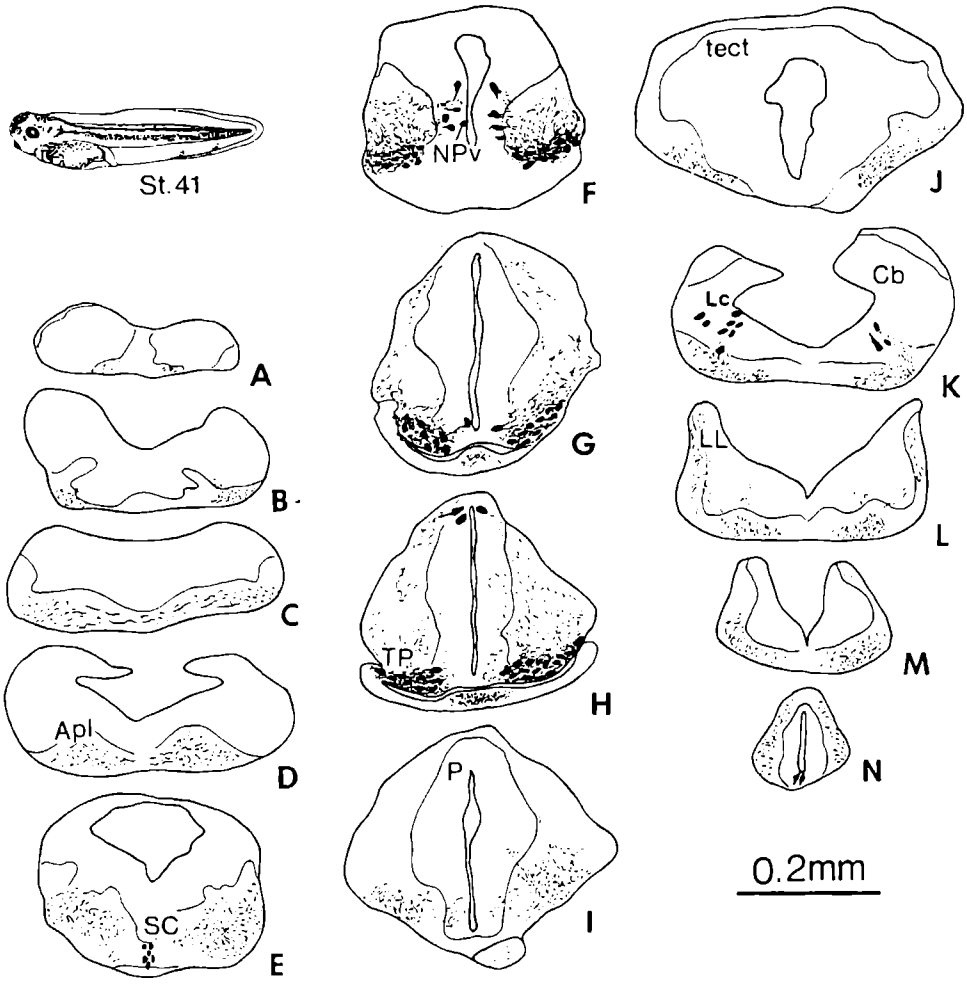


Fig. 2. A-N: Diagrams of transverse sections through the brain of *Xenopus laevis* at stage 41, from rostral (A) to caudal (N), showing the position of tyrosine hydroxylase-immunoreactive cell bodies (large dots) and fibers (small dots, wavy lines). *Apl*, lateral amygdala; *Cb*, cerebellum; *Lc*, locus coeruleus; *LL*, lateral line area; *NPv*, nucleus of the paraventricular organ; *P*, posterior thalamic nucleus; *SC*, suprachiasmatic nucleus; *TP*, posterior tubercle; *tect*, tectum mesencephali.

Premetamorphic stages

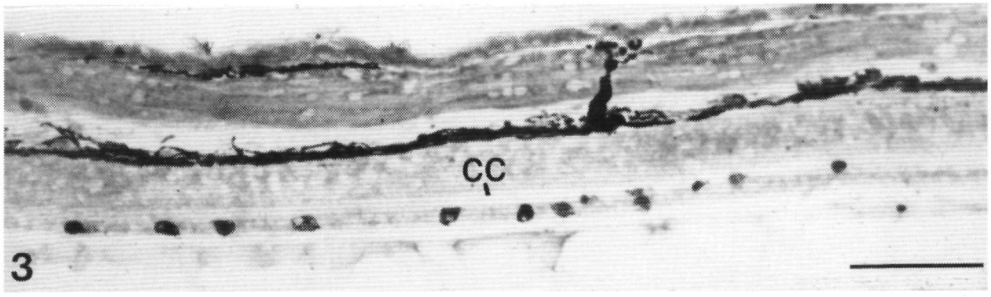
The development of catecholamine (CA) systems in the brain of *Xenopus* during premetamorphic stages is characterized by a progressive maturation of TH- and DA-immunoreactive cell bodies and fibers

From the beginning of this period, the immunoreactive cell bodies in the spinal cord display a more regularly spaced distribution and they are now more numerous at rostral spinal cord levels In the isthmic region, the TH-immunoreactive cells are clearly separated from the ventricle Their main processes are directed to the ventrolateral tegmentum, whereas other, thinner processes extend dorsally into the lateral aspect of the cerebellum

A new group of weakly stained TH-immunoreactive cells is, beginning with stage 51, observed in the proximity of the solitary tract In the hypothalamus, the cell groups gain individuality The TH- and DA-immunoreactive cell bodies in the suprachiasmatic nucleus can easily be subdivided into two subgroups, one in the midline beneath the ventricular tip, the other in the lateral part of the nucleus The medial subdivision contains cells that send short processes to the ventricular surface and are, therefore, considered to be liquor-contacting The TH- and DA-immunoreactive accompanying cells of the paraventricular organ have long processes that arborize extensively in the lateral parts of the diencephalon Also, the cell group in the posterior tubercle has matured considerably Not only the number of cells has increased, but also their location has undergone changes The cell group extends into the infundibular region Moreover, the cells that are located more medially reach the midline in the diencephalic-mesencephalic transition area A distinct, separate midbrain TH- and DA-immunoreactive cell group is not yet observed

At late premetamorphic stages (stages 52/53), TH- and DA-immunoreactive cells are also present in the posterior thalamic nucleus The cells are located near the ventricle and have processes that reach the lateral thalamus, the pretectal area, and even the midbrain tectum Another change is noted in the olfactory bulb, where the CA cells not only show an increasing laminar organization, but also display, starting with stage 49, DA-immunoreactivity

During the premetamorphic stages, a steady increase of the number of TH- and DA-immunoreactive cells in the retina occurs Compared to previous developmental stages, the cells still lie exclusively in the inner nuclear layer, but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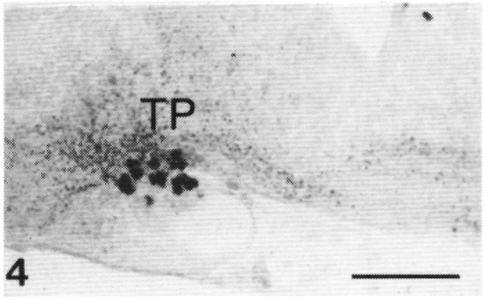
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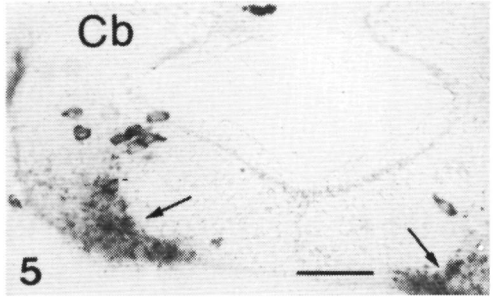
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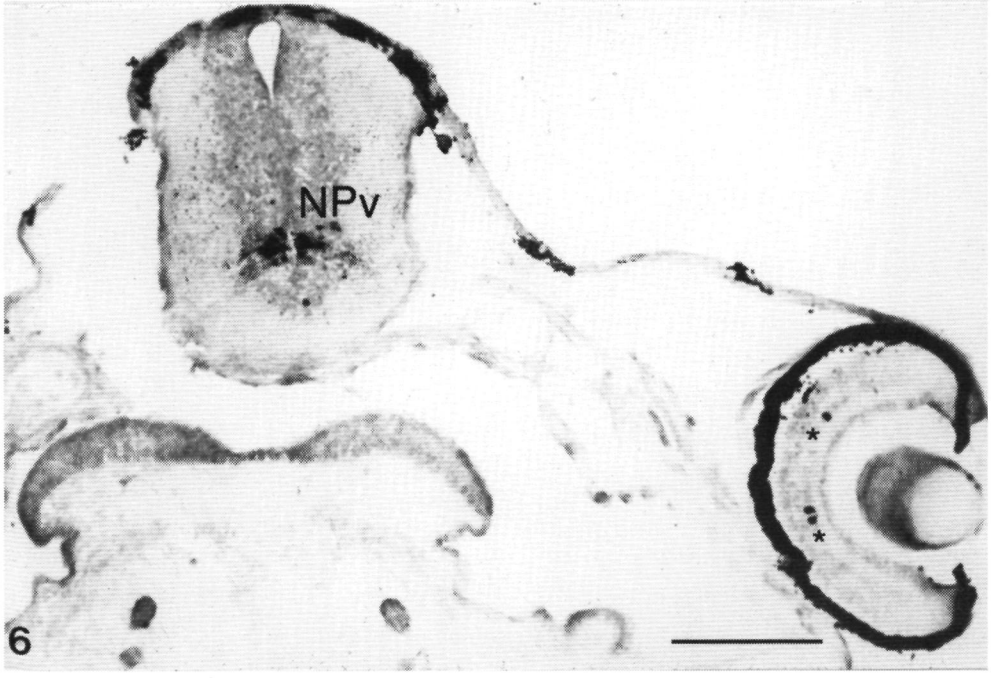
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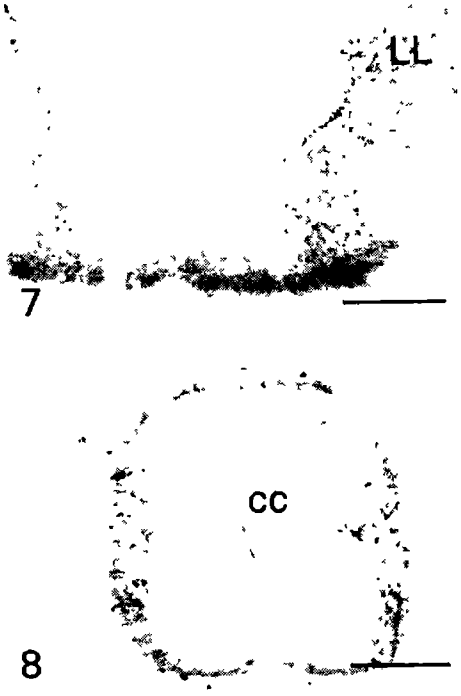
their processes are now laminarly arranged within the scleral and vitreal sublaminae of the inner plexiform layer.

With respect to the development of CA fiber systems during the premetamorphic stages, it should be noted that considerably denser TH- and



Figs 7 and 8. Photomicrographs of transverse sections through the brain of *Xenopus laevis* during premetamorphosis (stages 49-50) showing the distribution of TH-immunoreactive fibers in the rhombencephalon (**Fig. 7**) and the rostral spinal cord (**Fig. 8**). *cc*, central canal, *LL*, lateral line area Scalebar = 100 µm

DA-immunoreactive plexuses are found in the lateral line area (Fig. 7), the cervical and thoracic spinal cord segments (Fig. 8), and, starting from stage 51, in the nucleus accumbens and the striatum. Moreover, during the same period, immunoreactive fibers invade new targets, such as the torus semicircularis, tectum and thalamus.

Figs 3-6. Photomicrographs of sections through the brain and spinal cord of *Xenopus laevis* tadpoles at late embryonic stages **Figure 3** is a sagittal section showing dopamine-immunoreactive cell bodies ventral to the central canal (cc, stage 40) **Figure 4** shows in a sagittal section, DA-immunoreactive cells in the posterior tubercle (TP, stage 40), whereas **figure 5** documents the location of TH-immunoreactive cell bodies in the incipient locus coeruleus and the TH-immunoreactive longitudinal fiber tracts in the brainstem (arrows) at stage 41 **Figure 6** is a photomicrograph of a transverse section through the head (stage 43) showing DA-immunoreactive cell bodies in the nucleus of the paraventricular organ (NPv) and in the retina (asterisks) *Cb*, cerebellum Scalebar = 200 µm

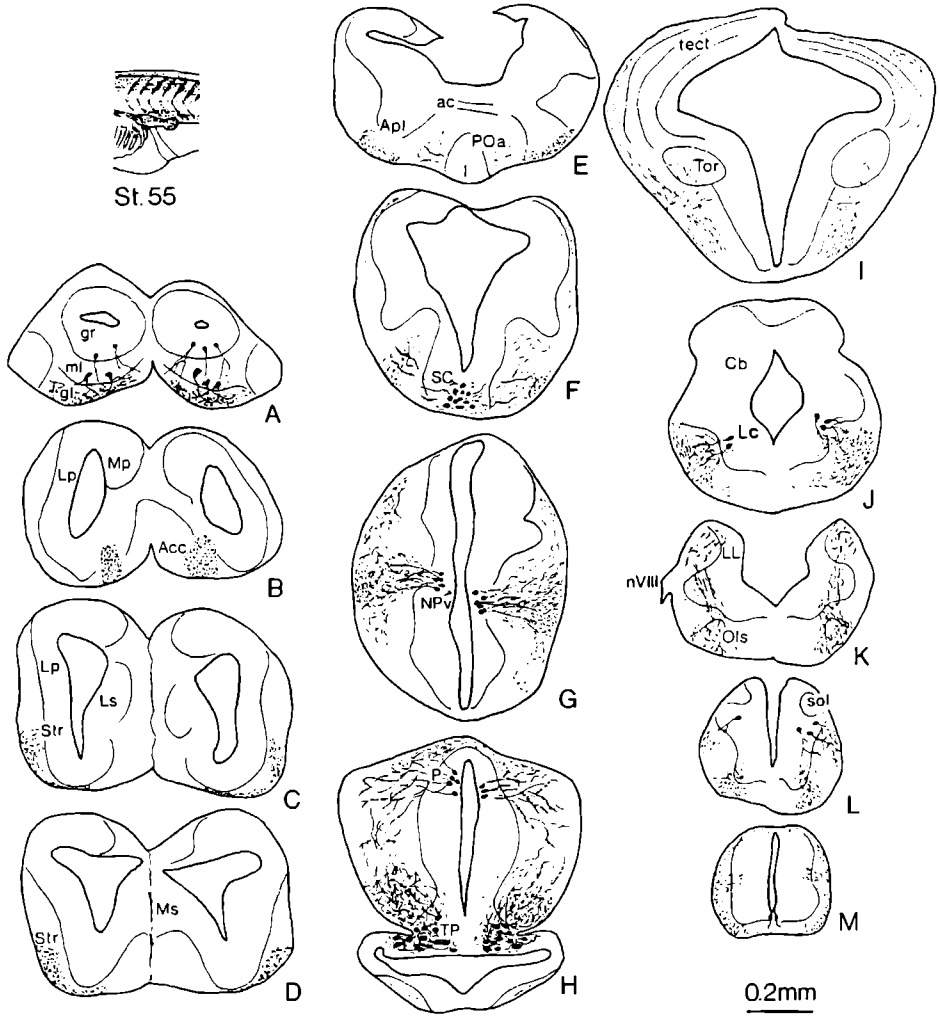


Fig. 9. A-M: Diagrams of sagittal and transverse sections through the brain of *Xenopus laevis* at prometamorphic stage 55 showing, from rostral (A) to caudal (M), the distribution of TH-immunoreactive cell bodies (large dots) and fibers (small dots, wavy lines). *ac*, anterior commissure; *acc*, nucleus accumbens; *Apl*, lateral amygdala; *Cb*, cerebellum; *gl*, glomerular layer of the olfactory bulb; *gr*, granular layer of the olfactory bulb; *LL*, lateral line area; *LP*, lateral pallium; *ml*, mitral layer of the olfactory bulb; *MP*, medial pallium; *Ms*, medial septum; *NPv*, nucleus of the paraventricular organ; *nVIII*, octaval nerve; *Ols*, superior olivary nucleus; *P*, posterior thalamic nucleus; *POa*, anterior preoptic nucleus; *SC*, suprachiasmatic nucleus; *sol*, solitary tract; *Str*, striatum; *tect*, tectum mesencephali; *Tor*, torus semicircularis; *TP*, posterior tubercle.

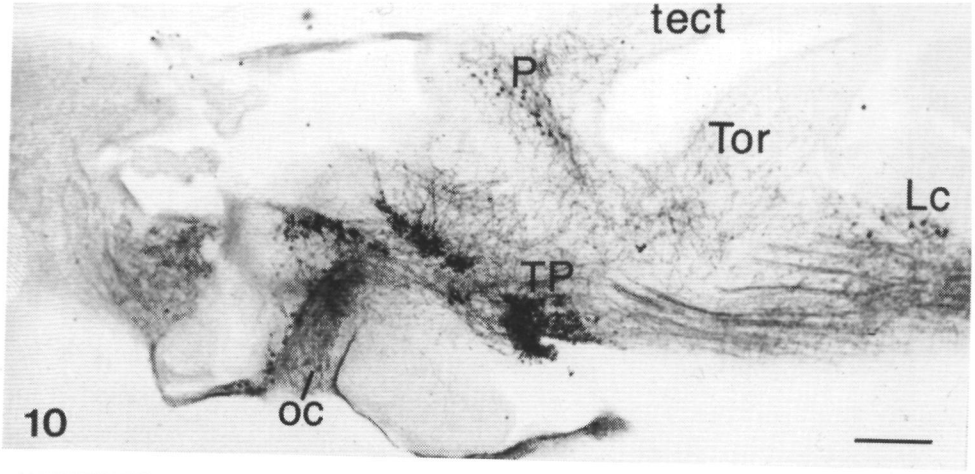
Prometamorphic stages

The prometamorphosis covers a rather long period of time during which the previously described CA cell groups and fiber plexuses mature primarily by increasing in number (Figs 9-16). The intensity of TH-immunoreactivity in the brains of prometamorphic larvae largely resembles that observed in the adult brain. In the hypothalamus, the cells in the posterior tubercle as well as those lateral to the paraventricular organ send long processes in dorsal and rostral directions (Figs 11,12), whereas processes of cells located in the suprachiasmatic nucleus can be traced to the median eminence and, further caudally, to the intermediate lobe of the hypophysis (Fig. 10). From stage 54 on, a separate group of TH- and DA-immunoreactive cells is found in the midbrain tegmentum (Fig. 10). In the olfactory bulb, the CA cells display a laminar organization with large, strongly TH/DA-immunoreactive cells located ventrally, around the glomeruli, and small, only weakly TH-immunoreactive cells in the granular layer (Figs 9,13,14). In the posterior thalamic nucleus, the TH- and DA-immunoreactive cells have long processes which extend into the lateral diencephalon where they intermingle with fibers arising from the hypothalamic CA cell groups (Fig. 9H). The isthmic group, *i.e.*, the locus coeruleus, has expanded rostrocaudally and lies dorsal to well-developed tracts that course longitudinally throughout the brainstem (Figs 9J,10).

TH-immunostaining of the few cells around the solitary tract is still weak during prometamorphic stages. A difference with previous stages, however, is that the cells are also weakly immunoreactive for DA antibodies from stage 55 on.

The distribution of TH- and DA-immunoreactive fibers in brains of premetamorphic larvae is almost identical to that observed in adult *Xenopus*. Well-developed plexuses of immunoreactive fibers occur in the rostral nucleus accumbens, striatum, septum, brainstem ventrolateral tegmentum, lateral line area and spinal cord (Figs 10-14).

As prometamorphosis proceeds, the dopaminergic neuronal elements in the retina reach a high degree of organization. Thus, at stage 57, numerous TH- and DA-immunoreactive cells are found in the inner nuclear layer. These cells show the typical morphology of amacrine cells with an extensive arborization of their processes into the vitreal and, in particular, scleral laminae of the inner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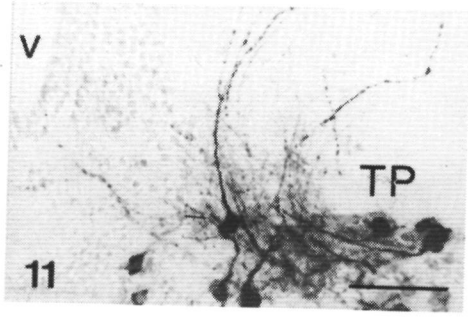
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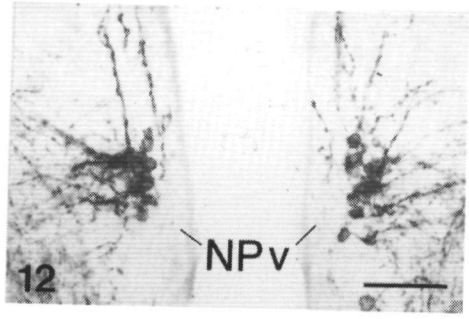
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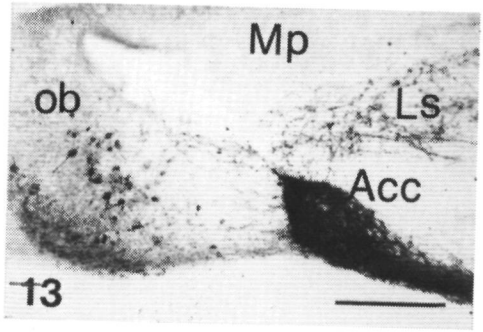
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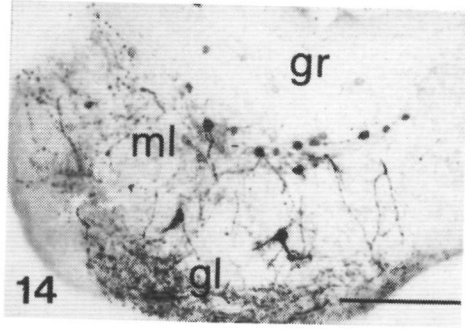
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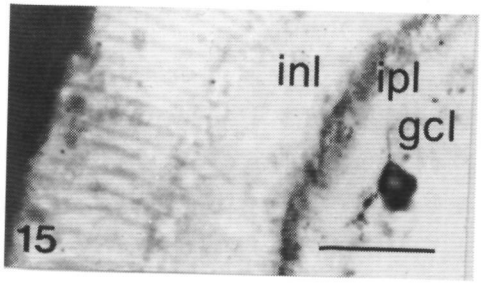
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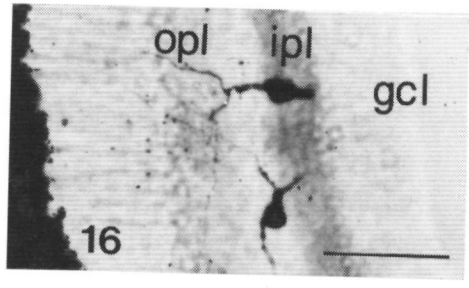
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plexiform layer. In addition, immunoreactive cells are present in the ganglion cell layer (displaced amacrine cells) with processes also branching within the inner plexiform layer (Fig. 15). Moreover, TH- and DA-immunoreactive interplexiform cells are found with processes extending not only to the inner plexiform layer, but also to the outer plexiform layer (Fig. 16).

Metamorphic climax

At the time the juvenile stages are reached, a basic pattern of CA organization is observed that is similar to that present in the adult brain (González et al., 1993). The brain grows in size and complexity and the CA systems achieve their final development. The major events that occur during these stages concern the maturation of the innervation of certain brain structures, such as the olfactory bulb, thalamus, midbrain tectum and torus semicircularis. Moreover, two new groups of TH- and DA-immunoreactive cell bodies appear during the metamorphic climax. One group, recognizable at stage 59, consists of CSF-contacting cells along the preoptic recess. The other group is a caudal continuation of the CA group around the solitary tract (now well stained with TH and DA antibodies) into the presumptive area postrema which is located dorsal to the central canal, immediately caudal to the obex. The cells in the area postrema are not found until the end of the metamorphosis.

Figs 10-16. Photomicrographs of sagittal and transverse sections through the brain of *Xenopus laevis* during prometamorphosis **Figure 10** shows in a sagittal section, the TH-immunoreactive cell bodies and fibers in the forebrain and rostral brainstem **Figure 11** and **figure 12** show, in transverse sections, the morphology of TH-immunoreactive cells in the posterior tubercle (TP) and lateral to the paraventricular organ (NPv) **Figure 13** is a photomicrograph of a sagittal section through the rostral telencephalon showing the dense TH-immunoreactive innervation of the nucleus accumbens and the immunoreactive cell bodies in the olfactory bulb **Figure 14** shows, in a transverse section, the disposition of TH-immunoreactive cells and fibers in the olfactory bulb **Figure 15** and **figure 16** illustrate a TH-immunoreactive displaced amacrine cell in the ganglion cell layer (**Fig. 15**) and interplexiform cells (**Fig. 16**) *ac*, anterior commissure, *acc*, nucleus accumbens, *gcl*, ganglion cell layer of the retina, *gl*, glomerular layer of the olfactory bulb, *gr*, granular layer of the olfactory bulb, *inl*, inner nuclear layer of the retina, *ipl*, inner plexiform layer of the retina, *Lc*, locus coeruleus, *Ls*, lateral septum, *ml*, mitral layer of the olfactory bulb, *Mp*, medial pallium, *Npv*, nucleus of the paraventricular organ, *ob*, olfactory bulb, *oc*, optic chiasm, *opl*, outer plexiform layer of the retina, *P*, posterior thalamic nucleus, *tect*, tectum mesencephali, *Tor*, torus semicircularis, *TP*, posterior tubercle, *v*, ventricle Scalebar = 200 μm in **figures 10** and **13**, 100 μm in **figures 11, 12** and **14**, and 45 μm in **figures 15** and **16**

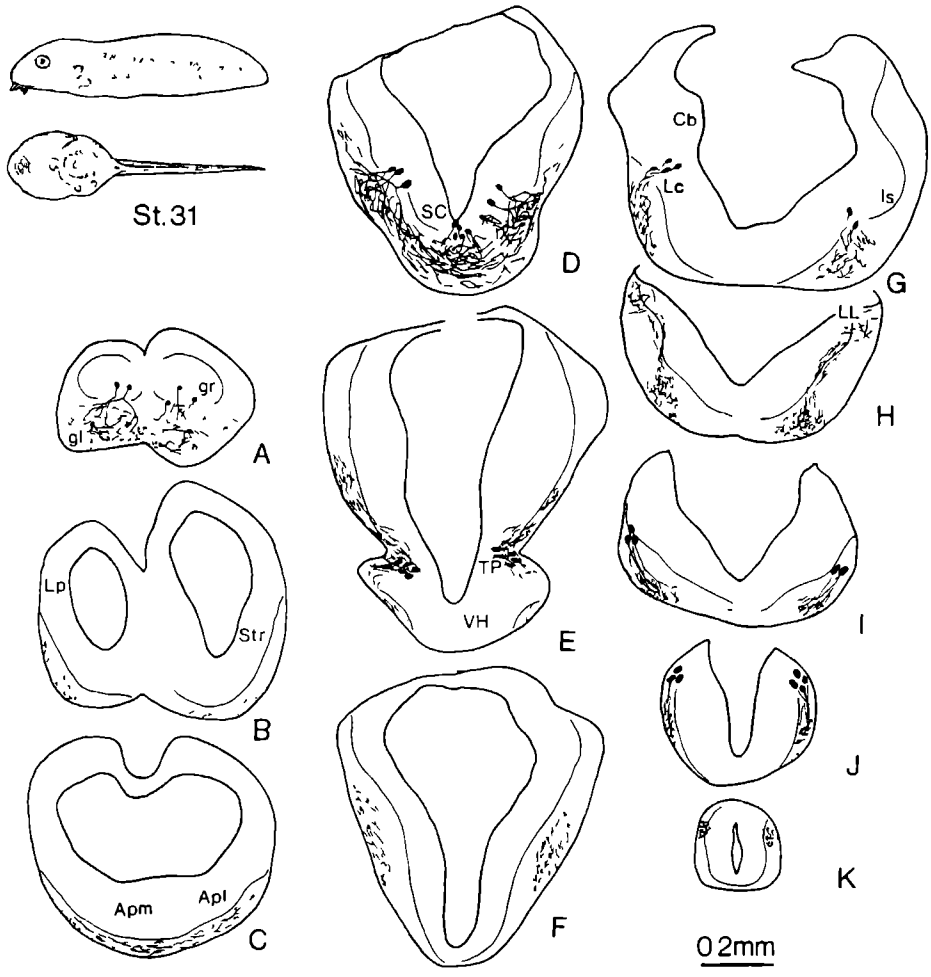


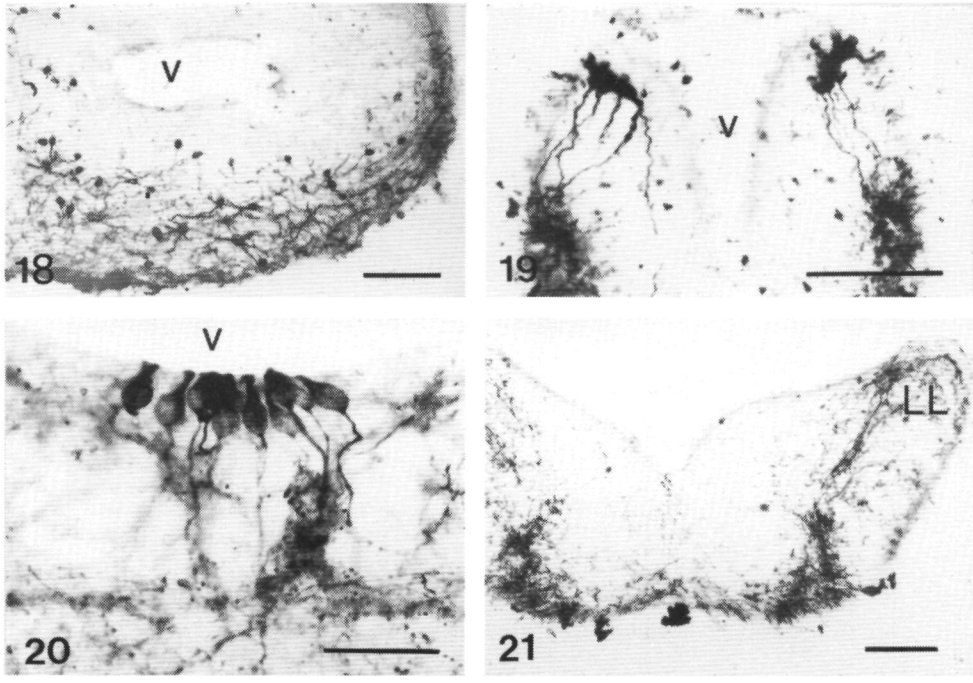
Fig. 17. A-K Diagrams of transverse sections through the brain of *Rana ridibunda* (stage 31) showing, from rostral (A) to caudal (K), the distribution of TH-immunoreactive cell bodies (large dots) and fibers (small dots, wavy lines) *apm*, medial amygdala, *apl*, lateral amygdala, *cb*, cerebellum, *gl*, glomerular layer of the olfactory bulb, *gr*, granular layer of the olfactory bulb, *Is*, isthmic nucleus, *Lc*, locus coeruleus, *LL*, lateral line area, *Lp*, lateral pallium, *SC*, suprachiasmatic nucleus, *Str*, striatum, *TP*, posterior tubercle, *VH*, ventral hypothalamic nucleus

The development of CA systems was also studied with TH antibodies in tadpoles of *Rana ridibunda* ranging from late embryonic up to juvenile stages Through the embryonic period, the CA systems develop progressively and at stage 31, just before the beginning of the larval period, six distinct TH-immunoreactive cell groups are observed As shown in figure 17, these groups are found in the olfactory bulb (Fig 18), suprachiasmatic nucleus, posterior tuberele up to the diencephalomesencephalic transition zone, isthmic region, solitary tract/area postrema (Fig 19), and ventral to the central canal of the spinal cord In particular, the early appearance of numerous cell bodies at caudal brainstem levels was noted

The distribution of TH-immunoreactive fibers in the brain of tadpoles at late embryonic stages is rather limited (Fig 17) Dense plexuses of immunoreactive fibers are found in the glomeruli of the olfactory bulb (Figs 17A,18), the ventral and ventrolateral portions of the diencephalon (Figs 17D,E), the ventrolateral tegmentum throughout the brainstem (Figs 17F-J,19), and the lateral line area A weak to moderate plexus is observed in striatal and amygdalar regions of the telencephalon (Figs 17B,C)

During the premetamorphic period (stages 32-39), no new groups of TH-immunoreactive cell bodies or fiber plexuses appear, but the maturation of CA systems proceeds by increase of the number of cells and fibers Particularly, cells similar to those beneath the central canal of the spinal cord are now present in larger numbers in the ependymal layer along the midline of the caudal rhombencephalon (Fig 20) The innervation of the nucleus accumbens, septum, thalamus, tectum and torus semicircularis is more elaborated In this period, the CA innervation of the lateral line area reaches its maximum (Fig 21)

In the subsequent prometamorphic period (stages 39-44/45), the distribution of TH-immunoreactive cell bodies and fibers shows more and more resemblance with the pattern observed in adults (Fig 22, see also Gonzalez and Smeets, 1991) Additional TH-immunoreactive cell groups are now observed in the posterior thalamic nucleus, midbrain tegmentum, and around the midline at caudal rhombencephalic levels In the infundibulum and among the cells lateral to the nucleus of the paraventricular organ, some TH-immunoreactive cells extend processes to the ventricular surface, suggesting a group of cells is present



Figs 18-21. Photomicrographs of transverse sections through the brain of *Rana ridibunda* tadpoles (stages 30-31) showing TH-immunoreactive cell bodies and fibers in the olfactory bulb (**Fig. 18**) and in the caudal brainstem, close to the obex (**Fig. 19**). **Figure 20** and **figure 21** correspond with premetamorphic tadpoles and show the well developed TH-immunoreactive cell group in the caudal rhombencephalon along the midline (**Fig. 20**) and the rich innervation of the lateral line area issueing from the longitudinal tracts (**Fig. 21**). *LL*, lateral line area; *v*, ventricle. Scalebar = 100 μm (**Figs 18, 19 and 21**) and 40 μm in **figure 20**.

which are separated but extend processes to the ventricular surface to contact the CSF (Fig. 23). However, cells in the nucleus of the paraventricular organ never express immunoreactivity for TH. In the isthmic region, the TH-immunoreactive cells of the locus coeruleus stain intensely and possess long thin processes that enter the cerebellum (Fig. 24).

Notable features of development of CA systems during the metamorphic

climax are the appearance of TH-immunoreactive cell bodies in the anterior preoptic area along the ventricular recess and the increase in the number of TH-immunoreactive fibers that innervate the lateral amygdala and the preoptic area (Fig. 25). Moreover, the TH-immunoreactive cell groups are better organized at these stages, as shown for the solitary tract / area postrema complex at the level of the obex (Fig. 26). On the contrary, a regression of the CA innervation of the rhombencephalic alar plate occurs. This reduction parallels the progressive loss of the lateral line system which takes place in this anuran species during metamorphosis.

DISCUSSION

A survey has been presented of the development of CA systems in the brain of two anuran species as demonstrated by immunohistochemical methods (Table 1). There are several aspects of this development that deserve comments. First, we discuss comparative aspects of development between amphibians. Subsequently, relationships between the development of CA systems and other developmental events in the CNS of amphibians are analyzed.

Comparative aspects of development of CA systems in the brain of amphibians

From the previous account, it is clear that the catecholamine systems in the two anuran species studied develop early during embryonic stages and they show a largely similar temporal sequence of appearance of CA neuronal elements. There are, however, a few differences between the species that deserve attention. First, whereas in tadpoles of *Xenopus* TH-immunoreactive cell bodies adjacent to the solitary tract are not seen until midpremetamorphosis, in *Rana* numerous TH-immunoreactive cell bodies are observed in a corresponding position already at the end of the embryonic period. Secondly, an earlier appearance of TH-immunoreactive cell bodies is also noted in the suprachiasmatic region of *Rana*. Moreover, cells in the olfactory bulb of *Rana* are strongly TH-immunoreactive early in development, whereas in *Xenopus* these cells are only weakly immunoreactive and do not show DA-immunoreactivity until premetamorphic stages. Because no data about DA-

immunoreactivity are available for *Rana*, it is currently unclear whether DA is already present in immunohistochemically detectable amounts at these early developmental stages.

When our results are compared with those of previous studies by means of the formaldehyde-induced fluorescence technique (Bartels, 1971; Terlou and Ploemacher, 1973; Notenboom, 1974; McKenna and Rosenbluth, 1975; Corio and Doerr-Schott, 1988), it becomes clear that TH immunohistochemistry enables an earlier detection of CA cell groups and fiber systems in amphibians. We found that these systems already start to organize at stages 39-41 (*Xenopus*) and 31-32 (*Rana*) which coincide with the moment the embryos start feeding, constantly swimming (without tactile stimulation), and using their gill structures, visual system and lateral line system. A recent study of the development of TH-immunoreactive cell groups and fibers in the diencephalon and mesencephalon of *Rana catesbeiana* during premetamorphic, prometamorphic and climax stages (Carr et al., 1990) revealed a similar sequence of appearance of TH-immunoreactive cell groups in those brain regions as reported by us. However, whereas we found a distinct TH- and DA-immunoreactive hypothalamohypophysial tract at early prometamorphic stages, Carr et al. (1990) were unable to identify immunoreactivity in that tract until the beginning of the climax period.

Temporal correlation between the development of CA systems and other developmental aspects of the CNS of anurans

As mentioned before, there are only a few studies that deal with changes in the CNS of anurans during development. The data are scarce and very diverse depending upon the point of interest of the authors. Nevertheless, in this section we attempt to correlate the data in the literature about developmental aspects of the CNS of anurans with events that take place, at the same time, in the development of the CA systems.

Spinal cord

A remarkable finding of the present study is the presence of numerous immunoreactive fibers in the marginal zone of the spinal cord of the two anuran

species studied.

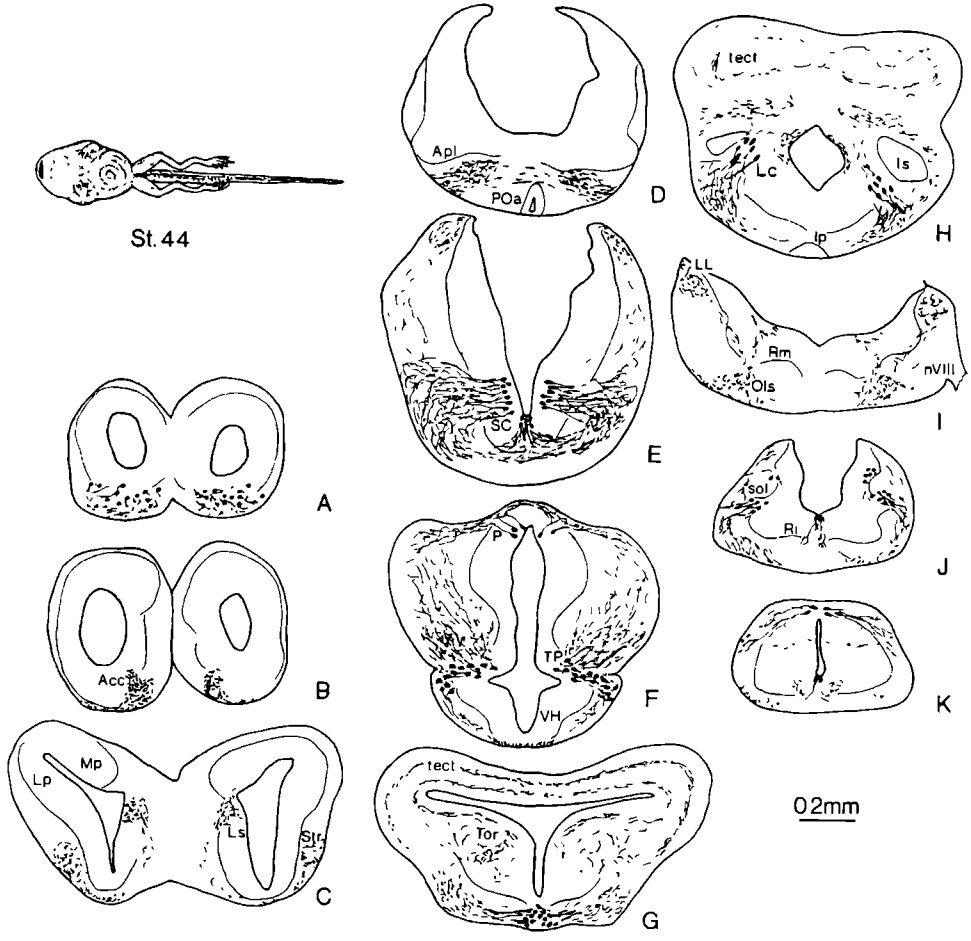


Fig. 22. A-K: Diagrams of transverse sections through the brain of *Rana ridibunda* at the end of the prometamorphosis (stage 44) showing, from rostral (A) to caudal (K), the distribution of TH-immunoreactive cell bodies (large dots) and fibers (small dots, wavy lines). *acc*, nucleus accumbens; *apl*, lateral amygdala; *Ip*, interpeduncular nucleus; *Is*, isthmic nucleus; *Lc*, locus coeruleus; *LL*, lateral line area; *Lp*, lateral pallium; *Ls*, lateral septum, *Mp*, medial pallium; *nVIII*, octaval nerve; *Ols*, superior olivary nucleus; *P*, posterior thalamic nucleus, *Poa*, anterior preoptic nucleus, *Rt*, inferior reticular nucleus; *Rm*, medial reticular nucleus; *SC*, suprachiasmatic nucleus; *sol*, solitary tract; *Str*, striatum; *tect*, tectum mesencephali; *Tor*, torus semicircularis, *TP*, posterior tubercle; *VH*, ventral hypothalamic nucleus.

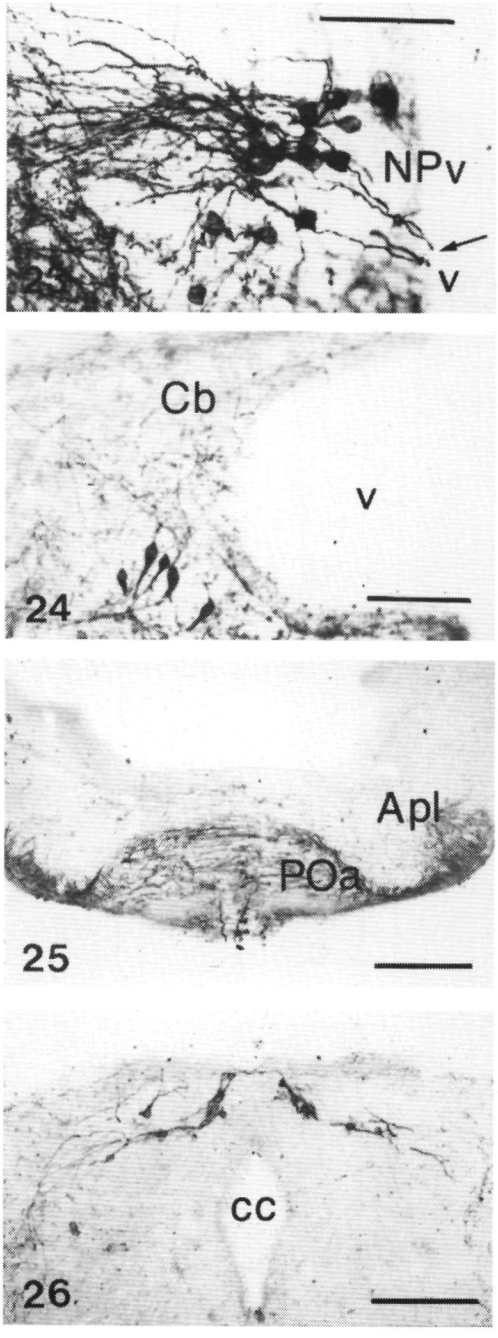
Even though the cell population ventral to the central canal of *Xenopus* has been shown to be immunoreactive for TH (Chen and Heathcote, 1992), it seems unlikely that the longitudinal TH-immunoreactive fibers arise exclusively from these cells. On the basis of the results of retrograde tracing studies, candidates of supraspinal CA input to the anuran spinal cord are the nucleus of the solitary tract, the locus coeruleus, the posterior tubercle and the preoptic nucleus (Ten Donkelaar et al., 1981; Tóth et al., 1985). However, the appearance of the longitudinal TH-immunoreactive fibers in the spinal cord preceeds that of TH-immunoreactive cells in the areas just mentioned, except for those in the posterior tubercle.

Moreover, developmental tract tracing studies have revealed that hypothalamospinal projections in *Xenopus* are not established until stage 57 (Ten Donkelaar and De Boer-Van Huizen, 1982), whereas projections arising from the locus coeruleus and the solitary tract nucleus are not present until stages 43 and 58/60, respectively (Van Mier and Ten Donkelaar, 1984; Norlander et al., 1985). A similar discrepancy may exist in ranid frogs (*cf.* Forehand and Farel, 1982; present study). Double labeling experiments with tract tracing techniques and immunohistochemistry are necessary for a better understanding of the origin of CA fibers to the spinal cord.

The early appearance of TH-immunoreactive fibers in the marginal zone of the spinal cord resembles the previously described serotoninergic supraspinal input (Soller, 1977; Van Mier et al., 1986) and suggests an involvement of both monoaminergic systems in early locomotor behavior or a role in differentiation, migration and maturation of spinal cord neurons at early developmental stages.

Rhombencephalic lateral line area

In most auran amphibians, *e.g.*, *Rana*, the neuromasts, afferents and second order cells of the brainstem that are related to the lateral line system, are lost during metamorphosis (Wahnschaffe et al., 1987). However, several anurans, including *Xenopus*, retain a functional lateral line system throughout life (Fritsch et al., 1984). The appearance of TH-immunoreactive fibers within the rhombencephalic lateral line area at early premetamorphic stages preceeds slightly the moment on which the lateral line nuclei can be recognized (Jacobi and Rubinson, 1983; *Rana catesbeiana*). The density of TH-immunoreactive



Figs 23-26. Photomicrographs of transverse sections through the brain of *Rana ridibunda* during prometamorphosis (**Figs 23,24**) and climax of the metamorphosis (**Figs 25,26**) showing TH-immunoreactive fibers in the cells lateral to the nucleus of the paraventricular organ (**Fig. 23**, arrow points to CSF-contacting processes) and in the locus coeruleus (**Fig. 24**). **Figure 25** shows the profuse innervation of the anterior preoptic area and the lateral amygdala, while **figure 26** illustrates the location of cells at the obex level. *Apl;* lateral amygdala; *Cb;* cerebellum; *cc,* central canal*;* *NPv*, nucleus of the paraventricular organ; *POa,* anterior preoptic nucleus; *v,* ventricle. Scalebar = 50 μm in **figure 23**, and 100 μm in **figs 24-26**.

fibers increases during the prometamorphosis in both *Rana* and *Xenopus*. Whereas in the latter species a distinct plexus of TH-immunoreactive fibers is present in the alar plate during the climax, in *Rana* the innervation of the lateral line area decreases in parallel with the loss of lateral line neurons and octaval cell proliferation (Kollros, 1981; Wahnschaffe et al., 1987).

Mesencephalon and diencephalon

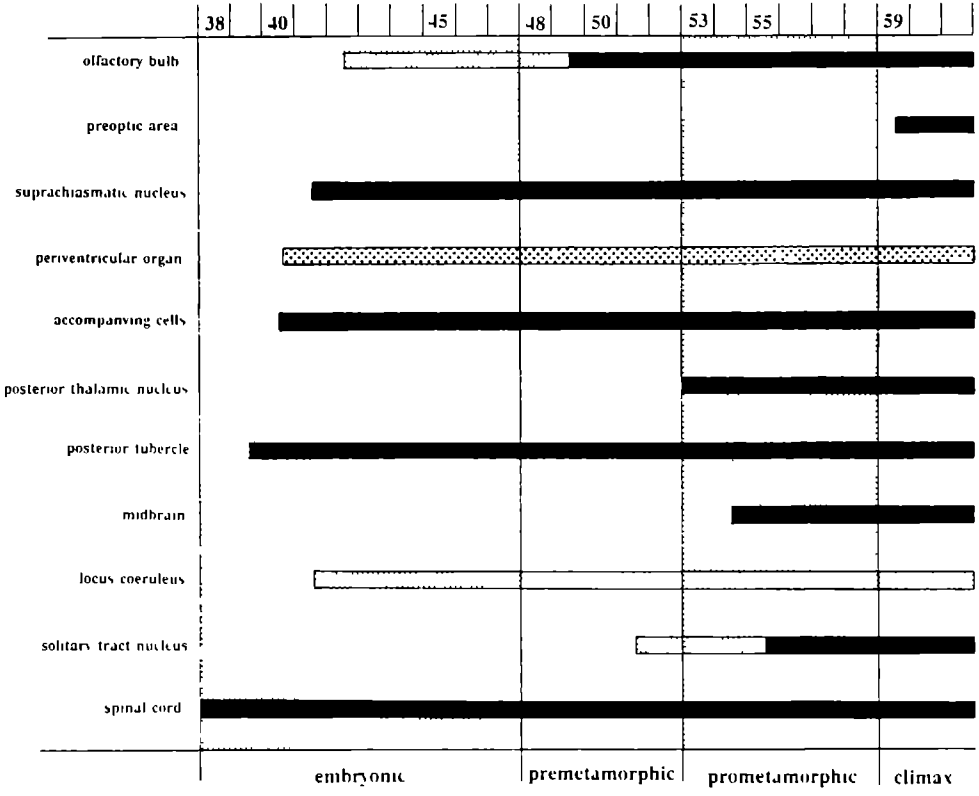
The development of catecholaminergic innervation of the midbrain tectum parallels that of tectal growth following a sequence from rostral to caudomedial (Straznicky and Gaze, 1972; Lewis and Straznicky, 1979). TH- and DA-immunoreactive fibers start to innervate lateral and superficial layers of the developing tectum and at the end of the premetamorphosis, when the pattern of retinotectal projections is established (Fawcett and Gaze, 1982), a laminar pattern of CA fibers is achieved at the same time.

The generation of diencephalic cells starts in the caudoventrolateral portion of the diencephalon and continues in a rostrodorsomedial direction (Clairambault, 1976; Tay and Straznicky, 1982). In the hypothalamus, the pre- and postchiasmatic parts display similar cytological characteristics but the prechiasmatic region does not differentiate until the end of the metamorphosis (Clairambault, 1976). The appearance of hypothalamic TH-immunoreactive cell bodies follows the same wave of development, beginning with the lateral aspect of the posterior tubercle followed by cell groups that lie more medially such as the accompanying cells of the paraventricular organ and the suprachiasmatic nucleus. In line with this pattern is also the relatively late appearance of TH- and DA-immunoreactive cells in the anterior preoptic area.

It is worth mentioning that the CSF-contacting cells in the hypothalamic paraventricular organ never express TH-immunoreactivity during development. These cells become strongly DA-immunoreactive at stage 40 without any trace of TH-immunoreactivity in preceding developmental stages. Previous studies (Smeets and González, 1990; González and Smeets, 1991; González et al., 1993) have shown that CSF-contacting cell bodies in the paraventricular organ of adult amphibians do stain for DA but not for TH. The results of the present study are in support of the notion made by Nakai et al. (1977) and Smeets et al. (1991) that these cell bodies do not synthesize DA themselves, but accumulate it from

the CSF. A likely source for the DA in the CSF could be the TH/DA-immunoreactive cell bodies in the spinal cord which are present, at least, already at stage 38.

Table 1 Timetable of appearance of CA cell groups in the CNS of *Xenopus laevis* from embryonic stage 38 through climax stage 61



Horizontal bars indicate the presence of cell groups containing only TH-immunoreactive cell bodies (small dots), TH- and DA-immunoreactive cell bodies (solid black) or cells that are exclusively DA-immunoreactive (large dots)

Probably the most remarkable finding of the present study is that the TH- and DA-immunoreactive cells in the midbrain of anurans seem to originate from the caudal hypothalamic cell group. It is unknown yet, whether the midbrain TH/DA

cells migrate out of the hypothalamus or constitute a continuous field of CA cells that spans the hypothalamus and the midbrain, with the hypothalamic ones maturing first. The time of appearance and the rostrocaudal distribution of cells favors the first suggestion. This finding may have consequences for our concepts of evolution of mesotelencephalic DA projections in vertebrates. It would imply that, despite the similarity in adult brains, the midbrain TH/DA cell groups of anamniotes and amniotes have different sites of origin which may reflect fundamental differences in basal ganglia organization.

A different conclusion is reached, however, when a segmental approach, as recently advocated by Puelles and Medina (1994), is used. Studying the development of CA systems in the brain of chicks, they noted that the ventral tegmental area DA cell group (A10) stretches across three segments (synencephalic, midbrain and isthmic). Similar observations were made by the same authors in the brains of pigeons and rats. Assuming that cell bodies generally do not cross the segmental and alar / basal boundaries, it seems likely that we have to reconsider the classification of CA cell groups in the brain of vertebrates. It is suggested, for example, that each segment gives rise to a part of the A10 group and that, within each segment, a part of the substantia nigra is formed by lateral migration. If so, it implies that the midbrain dopaminergic cell group of amphibians corresponds to the rostral part of that in the midbrain of amniotes (Puelles and Medina, 1994; Smeets and Reiner, 1994) and compared to amniotes, it appears rather late in development.

Telencephalon

As in the other brain regions, the ingrowth of TH-immunoreactive fibers into the telencephalon parallels the development of the hemispheres (*cf.* Clairambault, 1976). The first fibers innervating the telencephalon are found in amygdalar and striatal areas followed by the nucleus accumbens and the lateral septum. Curiously, in *Rana*, the fibers form initially a distinct neuropil external to the striatal cell plate, but in the metamorphic climax, the TH- and DA-immunoreactive fibers lie within the cellular plate, as is the case in the adult brain. In *Xenopus*, on the contrary, the TH- and DA-immunoreactive fibers stay external to the striatal cell layer.

Retina

Our study confirms the presence of TH-immunoreactive amacrine cells in the retina of amphibians as reported previously (Watt et al , 1988, Zhu and Straznicky, 1990,1991) It also shows that already at early stage 39 these cells are present in the inner nuclear layer of the retina of *Xenopus* and that they contain DA Moreover, our results reveal TH- and DA-immunoreactive putative displaced amacrine cells in the ganglion cell layer and interplexiform cells Similar observations have been made in a urodele, the tiger salamander *Ambystoma tigrinum*, by Yang et al (1991) The latter authors found that about 10% of the TH-immunoreactive cells lie in the ganglion cell layer, whereas 1% of the TH-immunoreactive cells of the inner nuclear layer possess interplexiform processes

Concluding remarks

As would be expected on the basis of the catecholamine biosynthetic pathway, detection of TH-immunoreactivity generally preceeds that of DA-immunoreactivity in developing dopaminergic cell groups It is, therefore, not surprising that, compared to fluorescence studies, an earlier appearance of several catecholamine cell groups and fiber system could be established Whereas a considerable number of studies describe the ontogeny of catecholamine systems in the CNS of mammals (for references, see Forster, 1993), relatively few studies deal with these systems in nonmammalian vertebrates (teleosts Ekstrom et al , 1992,1994, Manso et al , 1993, reptiles Medina et al , 1992a, birds Yurkewicz et al , 1981, Guglielmone and Panzica, 1984, 1985, Wallace et al , 1987, Puelles and Medina, 1994)

Although the number of developmental studies is limited, and different, sensitive techniques are used, it has become clear that, with respect to their first appearance, early, intermediate and late populations can be distinguished The posterior hypothalamic group, the locus coeruleus and the caudal brainstem group are the earliest to appear, but considerable variation exists between different classes of vertebrates, *e g* the relatively late appearance of CA cells in the locus coeruleus of reptiles (Medina et al , 1992a) and in the solitary tract nucleus of anurans (present study) and reptiles (Medina et al , 1992a) Since

immunodetection of catecholamines or their synthetic enzymes depends on the amount of these substances, the *in situ* hybridization techniques may be a welcome additional tool to investigate the development of CA systems The latter technique may also be more helpful in determining the delay between cell birth and the first expression of CA synthesis Nevertheless, there is presently ample evidence that some cells express CA immunoreactivity immediately after cell birth, whereas others become immunoreactive much later in their development For a better understanding of migratory processes, an early detection of future CA cells is a prerequisite

ACKNOWLEDGEMENTS

The authors are much indebted to Mrs B Jorritsma-Byham and Miss P G P Wismans for technical assistance, and to Mr D de Jong for preparing the photomicrographs The study was financially supported by NATO Collaborative Research Grant CRG 910970 and the Spanish DGICYTPB 90-0628

CHAPTER 3

The role of hypothalamic nuclei in the dopaminergic control of background adaptation in *Xenopus laevis*

With Eveline P. C. T. de Rijk, Ronnie G. P. Wismans, Wilhelmus J. A. J. Smeets and Eric W. Roubos

in Annals of the New York Academy of Sciences ***680;*** *486-488 (1993)*

Dopamine (DA) plays an important role in the inhibition of the secretion of α-melanophore stimulating hormone (α-MSH) from the melanotrope cells in the pars intermedia of the hypophysis (PI) of *Xenopus laevis* during adaptation of the animal to a white background (Jenks and Van Zoest, 1990). Dopamine coexists with neuropeptide Y (NPY) and γ-aminobutyric acid (GABA) in synapses on the melanotropes (De Rijk et al., 1992). The origin of this dopaminergic innervation has been studied with the formaldehyde-induced fluorescence technique (Terlou and Ploemacher, 1973) and micro-spectrofluorometry (Terlou and Van Kooten, 1974), using black-adapted *Xenopus* tadpoles. It was shown that fluorescence is present in the nucleus of the paraventricular organ (NPv), the posterior tubercle (TP) and the anterior preoptic nucleus (POa), indicating the presence of dopamine.

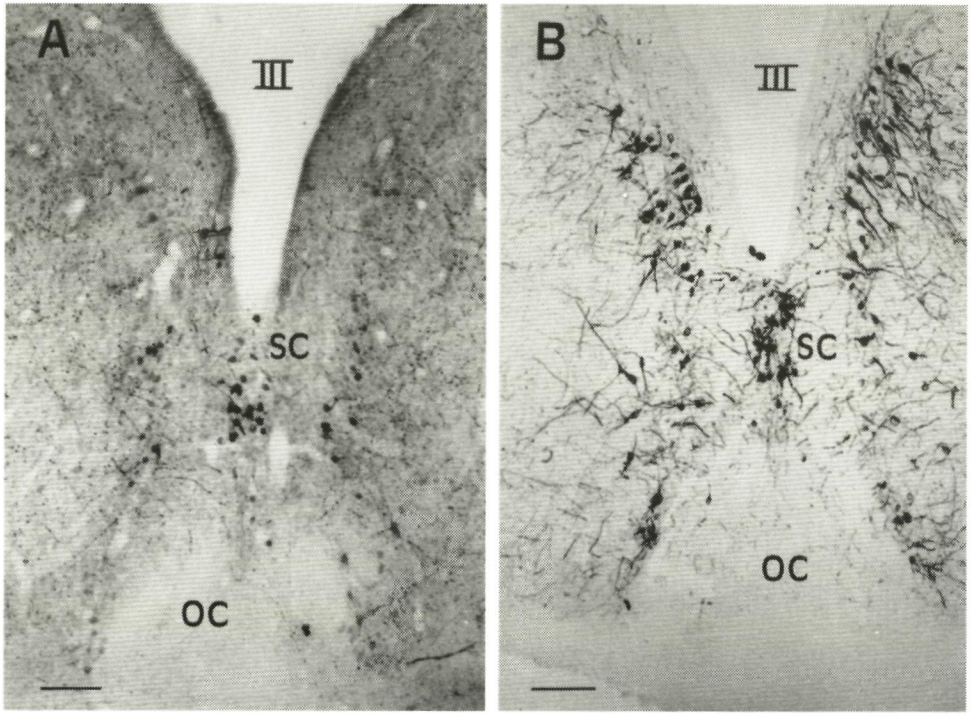


Fig. 1. Photomicrographs of transversal sections of the suprachiasmatic nucleus (SC) in adult *Xenopus laevis*, showing cell bodies and fibers stained with anti-DA (A) and anti-TH (B). The 15 μm thick freeze-microtome sections were stained according to the PAP method. The DA antiserum was diluted 1:3000, anti-TH 1:1000. *III*, third ventricle; *OC*, optic chiasm; *SC*, suprachiasmatic nucleus. Scalebar = 100 μm.

Based on the course of the fluorescent fiber tracts to the pars intermedia, the nucleus of the paraventricular organ was considered to be involved in the regulation of the melanotrope cells (Terlou and Ploemacher, 1973; Terlou and Van Kooten, 1974).

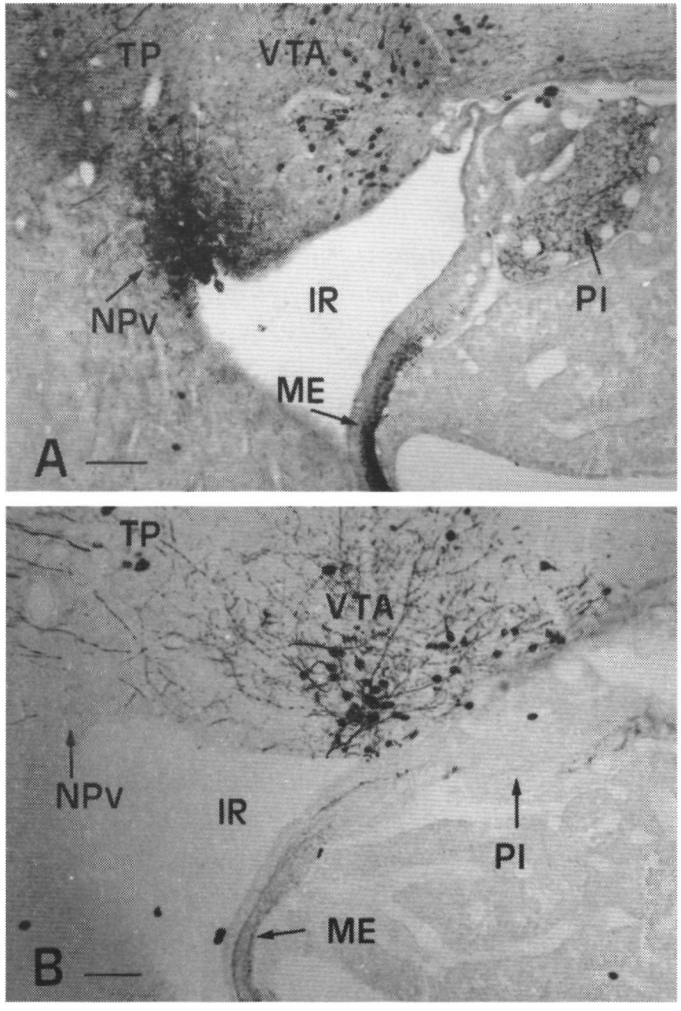


Fig. 2. Low power micrographs of sagittal sections of the infundibular region in adult *Xenopus laevis*, showing immunoreactive cell bodies in the nucleus of the paraventricular organ (NPV) with the DA-antiserum (A), and the lack of immunostaining in the same organ with anti-TH (B). The posterior tubercle (TP), ventral tegmental area (VTA), median eminence (ME), and pars intermedia (PI) are positive with both antisera. *IR*, infundibular recess. Scalebar = 100 μm.

The aim of the present study was to reinvestigate the putative sources of the dopaminergic control of background adaptation in *Xenopus laevis* by means of immunohistochemical methods Antisera against dopamine and the enzyme tyrosine hydroxylase revealed immunoreactive cell bodies in the posterior tubercle, the anterior preoptic nucleus, the suprachiasmatic nucleus (Fig 1) of both larval and adult *Xenopus laevis* The nucleus of the paraventricular organ also contains dopamine-positive neurons (Fig 2A), but they are, in contrast to the other dopamine-containing nuclei, tyrosine hydroxylase-negative (Fig 2B) This indicates that these cells are not able to synthesize dopamine

Dopamine and tyrosine hydroxylase-positive fibers were seen running underneath the ventral infundibular nucleus to terminate in the pars intermedia The cell bodies of the fibers seem to be situated in either the posterior tubercle or the suprachiasmatic nucleus Co-existence of dopamine, neuropeptide Y and γ-aminobutyric acid in the terminal network of the pars intermedia ((De Rijk et al , 1992) and the co-existence of these three transmitters in the suprachiasmatic nucleus makes this nucleus the most likely candidate for controlling the pars intermedia (Tuinhof et al , 1992) Moreover, immunocytochemical staining intensity with anti-neuropeptide Y was clearly different in the suprachiasmatic nucleus under different conditions of background adaptation (Tuinhof et al , 1993b) A central role of the suprachiasmatic nucleus in the control of α-MSH from the pars intermedia during background adaptation fits well with the existence of a direct connection between retinal fibers and the preoptic nuclei including the suprachiasmatic nucleus in the frog *Rana temporaria* (Vullings and Van Heussen, 1975) and the presence of dopamine, tyrosine hydroxylase (Smeets and González, 1990, Carr et al , 1991, González and Smeets, 1991), γ-aminobutyric acid (Franzoni and Morino, 1989) and neuropeptide Y (Danger et al , 1985) in the suprachiasmatic nucleus of frogs (*Rana catesbeiana*, Carr et al , 1991, *R esculenta*, Franzoni and Morino, 1989, *Rana ridibunda*, Danger et al , 1985, Smeets and Gonzalez, 1990, Gonzalez and Smeets, 1991)

CHAPTER 4

Neuropeptide Y in the developing and adult brain of the South African clawed toad *Xenopus laevis*

With Agustin González, Wilhelmus J.A.J. Smeets and Eric W. Roubos

in Journal of Chemical Neuroanatomy 7; 271-283 (1994)

ABSTRACT

To get more insight into developmental aspects of neuropeptide Y (NPY)-containing neuronal structures in the brain of amphibians and their possible involvement in background adaption, we have studied immunohistochemically the distribution of this neuropeptide in embryos, larvae and adults of *Xenopus laevis* Antisera against NPY revealed that already at early embryonic stages NPY immunoreactive cell bodies are present in the ventral thalamus and rhombencephalic tegmentum Slightly later, cell bodies appear in the olfactory bulb, the basal forebrain including the lateral and medial amygdala, the preoptic area, the ventral and dorsal thalamus, the suprachiasmatic region, the anteroventral tegmental nucleus and the solitary tract area At late embryonic stages, the NPY cell groups show not only an increase in number of cells, but also stain more intensely Around the time of hatching, a dramatic decrease in the number of immunodetectable cells occurs, particularly in the basal forebrain and in the rhombencephalic tegmentum At the same time, however, new cell groups appear in telencephalic pallial regions and in the torus semicircularis By the end of the premetamorphic stages, the distribution of NPY-immunoreactive cell bodies and fibers resembles closely the pattern observed in adult *Xenopus* brains When compared to the development of catecholamine systems, it is clear that the NPY neurotransmitter system develops earlier However, the expression of NPY- and dopamine-immunoreactivity in the suprachiasmatic nucleus occurs at about the same time (around stage 40) and coincides with several other events which are related to background adaptation suggesting that this nucleus plays a key role in this complex neuroendocrine mechanism

INTRODUCTION

Recent studies of the complex neuroendocrine mechanism that enables the South African clawed toad *Xenopus laevis* to adapt physiologically to the light intensity of the background by changing the color of the skin, have revealed that neuropeptide Y (NPY) may play an important role in background adaptation (Jenks et al , 1993, Roubos et al , 1993, Tuinhof et al , 1993b) With *in vitro* superfusion studies it has been shown that NPY, together with dopamine (DA) and GABA, inhibits the release of melanophore-stimulating hormone (α-MSH), which causes

the dispersion of melanin in the skin melanophores (Verburg-Van Kemenade et al., 1986a,b, 1987a; Jenks et al., 1993). Immunoelectron microscopy revealed that the three neurotransmitters coexist in nerve terminals that make synaptic contacts with the melanotrope cells in the intermediate lobe of the hypophysis (De Rijk et al., 1990a, 1992). With tract-tracing techniques it was demonstrated that perikarya of the suprachiasmatic nucleus project to the intermediate lobe (Tuinhof et al., 1994a). Further support for the notion that the suprachiasmatic nucleus could play a major role in the control of background adaptation was obtained by immunocytochemical and *in situ* hybridization experiments (Tuinhof et al., 1993b). Finally, preliminary results provide evidence that in some cell bodies of the suprachiasmatic nucleus DA and NPY coexist (Tuinhof et al., 1994b).

In addition to the studies of brain centers containing DA, NPY and GABA in the adult *Xenopus*, we have recently started research on development of these centers in order to provide more insight into the functional significance of these neurotransmitters in the brain in general and in the background adaptation controlling system in particular. First, the development of catecholamine systems has been studied by means of antibodies against tyrosine hydroxylase (TH) and DA (González et al., 1994). It was found that already in late embryonic stages (40/41 according to the timetable of development by Nieuwkoop and Faber, 1967), DA immunoreactive cells were present in the posterior tubercle, the nucleus of the paraventricular organ and its accompanying cells, and in the suprachiasmatic nucleus. At about the same time, TH-immunoreactive cell bodies were observed in the isthmic region. From stage 41 onwards, TH- and DA-immunoreactive fibers can be traced to the hypophysis.

The aim of the present study is to provide similar information on the development of NPY-immunoreactive structures. To evaluate the results, a comparison has been made with the situation in adults. The presence of NPY in the brain of adult amphibians has been described extensively for the frog *Rana* (Danger et al., 1985; Lázár et al., 1993), but information about the distribution of NPY in the brain of *Xenopus laevis* has been dealt with in less detail (Lázár et al., 1993; Tuinhof et al., 1993b). Therefore, the present developmental study includes a description of NPY-distribution in adult *Xenopus* with reference to previous data on *Xenopus* and *Rana*.

MATERIALS AND METHODS

About fifty *Xenopus laevis* tadpoles, ranging from developmental stage 32 to stage 65, and six adult specimens, reared in the laboratory of the department of Animal Physiology, Nijmegen, were used. *Xenopus* larvae were obtained by Pregnyl-induced (Organon, Oss, The Netherlands) breeding and kept in tap water at 20-25 °C throughout their development. The larvae were raised on nettle powder or canned spinach, whereas older larvae and young froglets were fed Tubifex. At appropriate times, embryos and tadpoles were anaesthetized in a 0.3% solution of tricaine methanesulfonate (MS 222, Sandoz, Basel, Switzerland) and subsequently fixed by immersion in Bouin's fluid. Adult animals were also anaesthetized in MS 222, but then transcardially perfused with 75 ml of Ringer's solution followed by 150 ml 4% paraformaldehyde in 10 mM sodium phosphate buffer (pH 7.6). Dissected brains were postfixed in the same fixative for 24 hours.

Immunocytochemistry

Tissues were rinsed and cryoprotected in 30% sucrose in sodium phosphate buffer (10 mM, pH 7.6). Cryostat cut 25 µm sections were transferred to poly-L-lysine-coated slides and dried at 20 °C. Tris-buffered saline (TBS; 50 mM, pH 7.6) containing 150 mM NaCl and 0.3% Triton (TBS-TX; Sigma, St Louis, MO, U.S.A.) was used as solvent for the antibodies and as rinsing fluid between incubations.

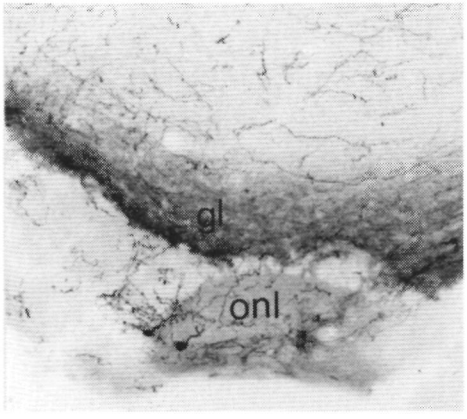
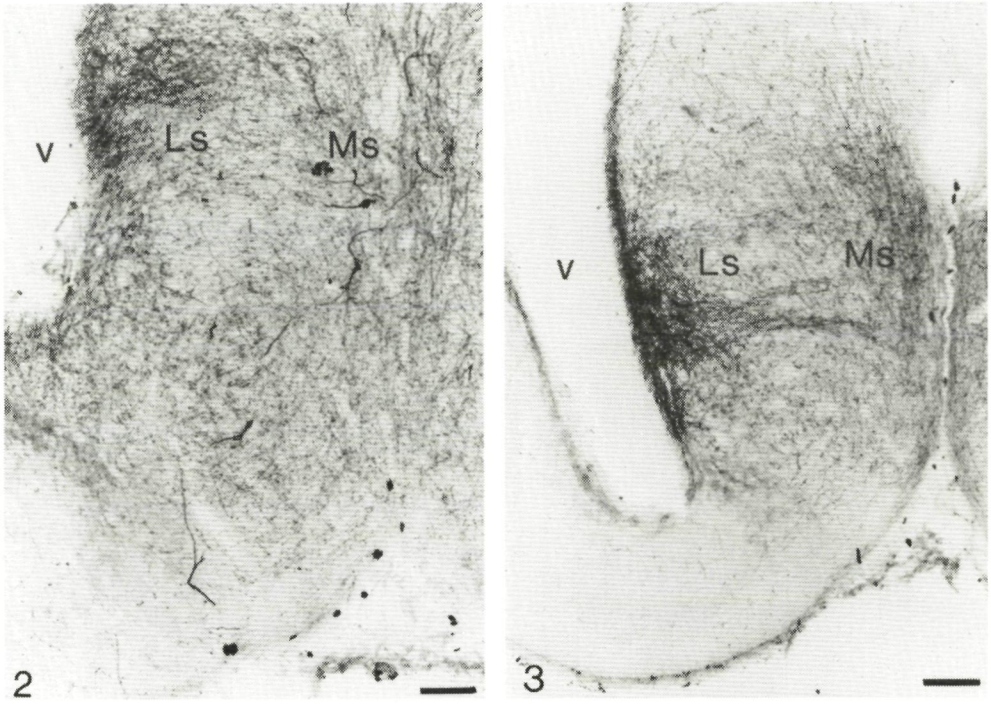


Fig. 1. Transverse section through the rostral pole of the main olfactory bulb of an adult *Xenopus* showing some Golgi-like stained NPY-immunoreactive cell bodies in the olfactory nerve layer (onl) and a rather dense plexus of fibers in the glomerular layer (gl). Scalebar = 100 µm.

Sections were rinsed in TBS-TX (30 min) and incubated for 30 min in 20% normal goat serum to prevent aspecific binding. Then anti-NPY (1:4000; gift from Dr H. Vaudry) was applied for 72 hrs at 4 °C. Goat-antirabbit second antiserum (1:50; Nordic, Tilburg, The Netherlands) was applied for 1.5 hr at 20 °C. Finally, the sections were incubated in rabbit peroxidase-antiperoxidase (1:1000; Nordic), for 1.5 hr at 20 °C. Rinsing in TBS-TX and TBS preceded visualization of reaction product with 0.04% 3,3' diaminobenzidine (Sigma), 0.25% nickel ammonium sulphate and 0.015% H_2O_2 in TBS. The reaction was terminated by rinsing in TBS. Finally, the sections were dehydrated and embedded in Entellan.

For the adults, a slightly different procedure was used which included the following steps: (1) incubation in rabbit anti-NPY serum (gift from Dr J.D. Mikkelsen), diluted 1:1,000 for 36 hrs; (2) incubation in swine anti-rabbit serum



Figs 2 and 3. Transverse sections through the forebrain of an adult *Xenopus* showing darkly stained NPY-immunoreactive cell bodies in the medial septum and dense plexuses of fibers in both the medial and lateral septum. *Ls*, lateral septum; *Ms*, medial septum; *v*, ventricle. Scalebar = 100 μm.

(Nordic), diluted 1:50 for 1 hr; and (3) incubation in rabbit peroxidase-antiperoxidase (Dakopatts, Copenhagen, Denmark) diluted 1:800 for 1 hr. For details about the specificity of the NPY antisera applied to the brain of developing and adult *Xenopus*, the reader is referred to the studies by Danger et al. (1985) and Blinkenberg et al. (1990), respectively.

RESULTS

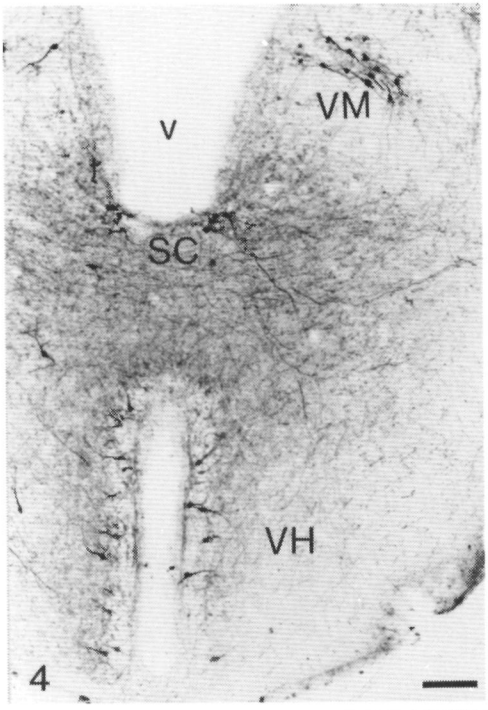
NPY-immunoreactivity in adult *Xenopus* brain

In all adult brains used for the present study, staining with NPY antisera revealed a similar pattern. Whereas fiber staining was always very distinct, only varying in density from place to place, considerable variation occurred in the staining of cell bodies throughout the brain. Three degrees of cell staining were distinguished, namely light, moderate and dark.

Olfactory bulb

At very rostral levels of the main olfactory bulb, a few, Golgi-like stained immunoreactive cells were found within the olfactory nerve layer (Fig.1). The processes of these cells, however, cover most of the layer and extend into the glomerular layer. More numerous, but only lightly stained cells were observed within the internal granular layer. Immunoreactive fibers are present in all layers of the main olfactory bulb, but predominantly in the glomerular layer and the external plexiform layer.

Figs 4-7. Transverse sections through the diencephalon and rostral midbrain of an adult *Xenopus* showing darkly stained NPY-immunoreactive cell bodies in the ventromedial thalamic nucleus (**4, 5**), the suprachiasmatic nucleus and the ventral hypothalamic nucleus (**4, 6**), and in the anteroventral tegmental nucleus of the midbrain (**7**) **Figures 5** and **6** are higher magnifications of **figure 4** *Av*, anteroventral tegmental nucleus, *SC*, suprachiasmatic nucleus, *v*, ventricle, *VH*, ventral hypothalamic nucleus, *VM*, ventromedial thalamic nucleus, Scalebar = 100 μm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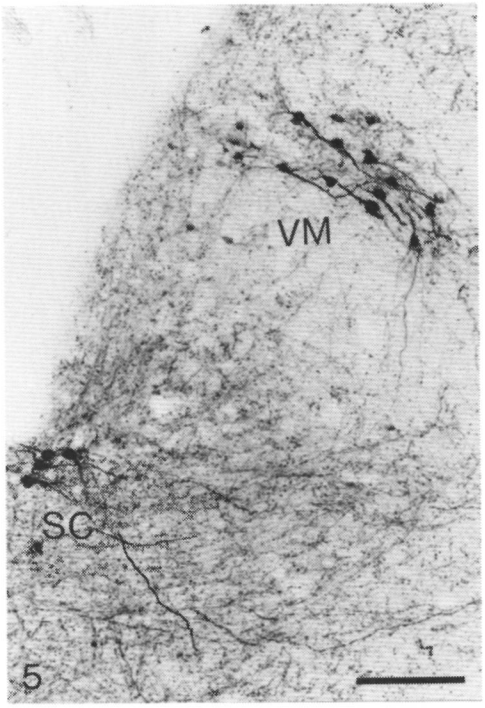
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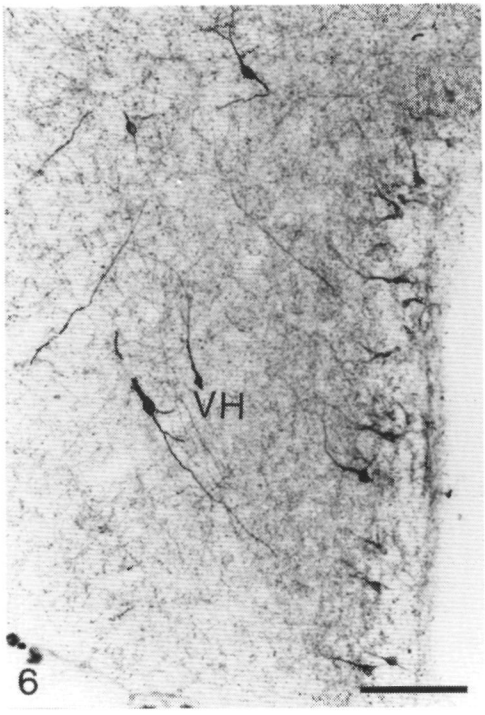
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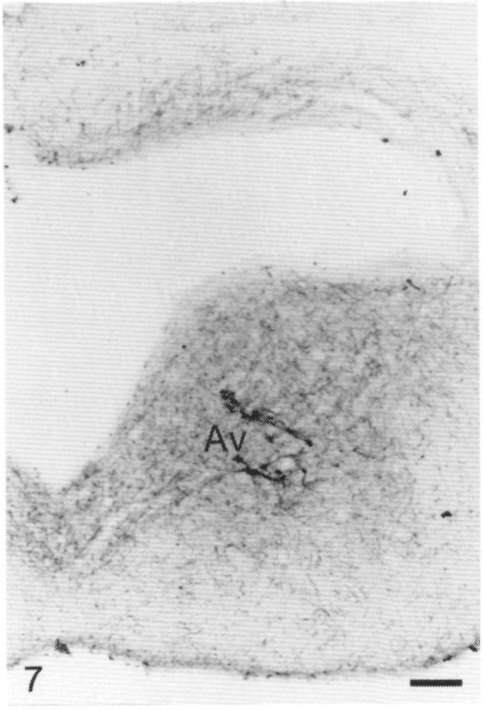
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In sharp contrast to the situation found in the main olfactory bulb, the accessory olfactory bulb does not show any sign of immunoreactivity.

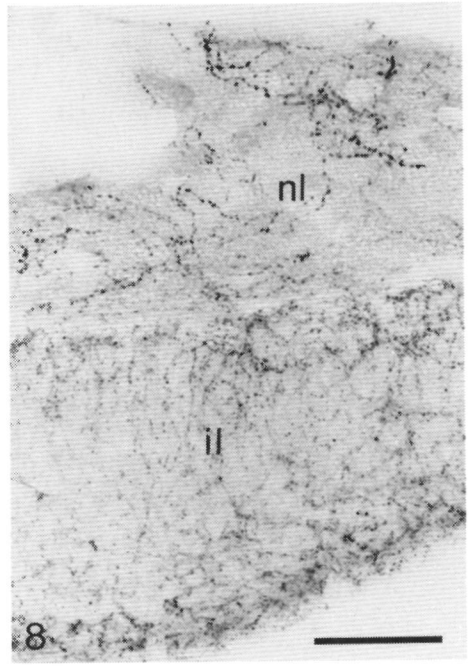
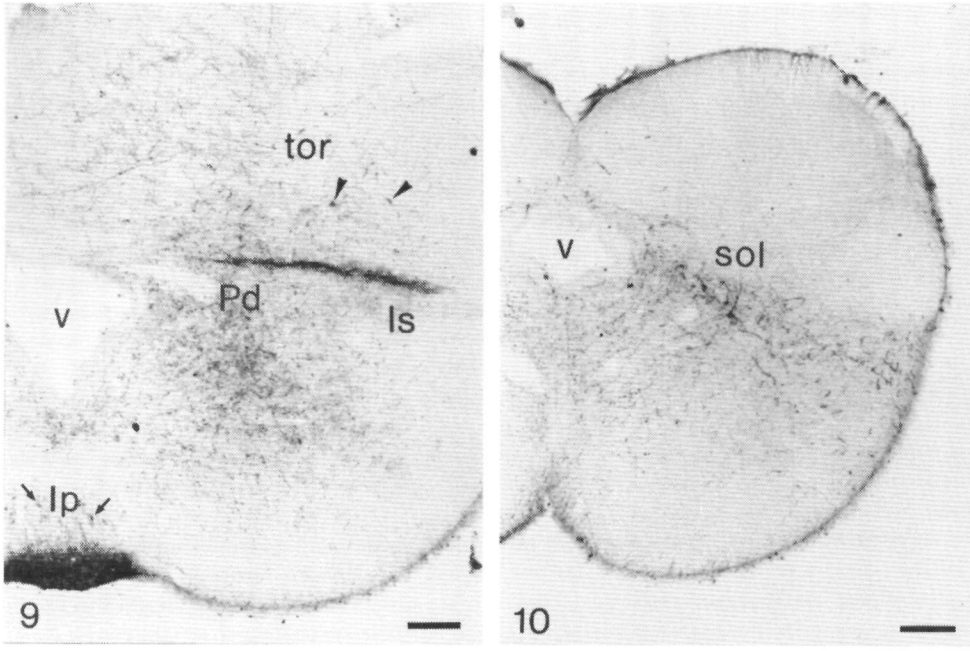


Fig. 8. Transverse section through the hypophysis of an adult *Xenopus* showing NPY-immunoreactive fibers in the neural and intermediate lobes. Note the generally larger diameter of the varicose fibers in the neural lobe. *il*, intermediate lobe of the hypophysis; *nl*, neural lobe of the hypophysis. Scalebar = 100µm.

Telencephalon proper

NPY-immunoreactive cell bodies are widely spread throughout the telencephalon proper. Lightly and moderately stained cell bodies occur in the lateral, dorsal and medial pallial areas and in the lateral amygdala, whereas darkly stained cells are present in the medial septum (Fig. 2), the medial amygdala and the lateral preoptic area. Immunoreactive fibers are also widely spread and a particularly dense innervation is found in the nucleus accumbens, the lateral and medial septum (Figs 2,3), the lateral amygdala and the preoptic region.

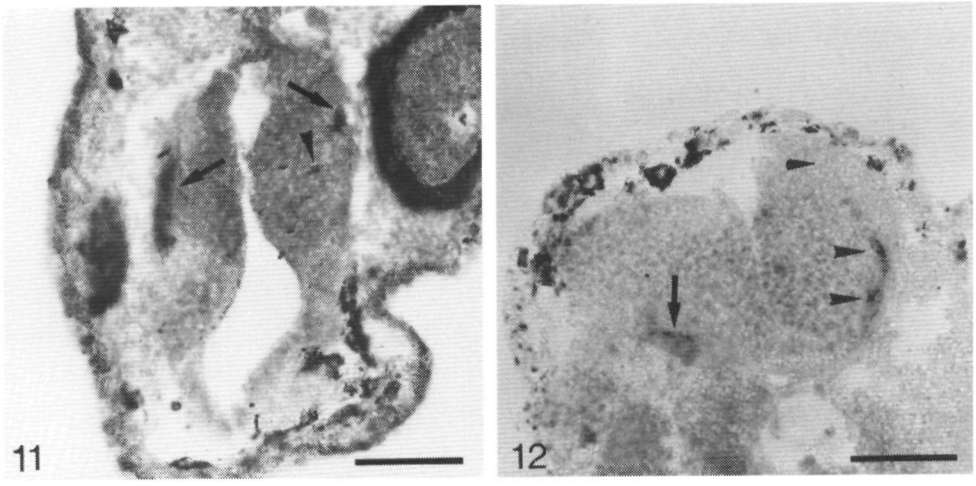


Figs 9 and 10. Transverse sections through the isthmic region (**9**) and caudal brainstem (**10**) of an adult *Xenopus*. Note the lightly stained cells in the magnocellular nucleus of the torus semicircularis (arrowheads) and in the interpeduncular nucleus (arrows). Also note the darkly stained, band-like plexus of NPY-immunoreactive fibers between the torus dorsally and the posterodorsal tegmental nucleus and the isthmic nucleus ventrally, as well as the equally dense plexus ventral to the interpeduncular nucleus. *Ip*, interpeduncular nucleus; *Is*, isthmic nucleus; *Pd*, posterodorsal tegmental nucleus; *sol*, solitary tract; *tor*, torus semicircularis; *v*, ventricle. Scalebar = 100 μm.

Diencephalon.

The majority of immunoreactive cell bodies lies within the diencephalon. Numerous darkly stained cell bodies were found in the suprachiasmatic nucleus, the ventromedial thalamic nucleus, and in the ventral hypothalamic nucleus (Figs 4-6). Additionally, a considerable number of lightly and moderately stained cells occur in the posterior thalamic nucleus, the ventrolateral part of the posterior tubercle and in the ventral hypothalamic nucleus. Most diencephalic regions contain dense plexuses of immunoreactive fibers (Figs 4-6). Thick-beaded and thin-beaded varicose fibers are readily identifiable. Immunoreactive fibers can be

traced via the median eminence to the hypophysis where they enter the neural and, especially, the intermediate lobe (Fig. 8). A difference was observed with respect to the morphology of the fibers in these lobes. The immunoreactive fibers in the neural lobe are predominantly thick with large beads, whereas those in the intermediate lobe generally appear thinner with rather small beads.



Figs 11 and 12. Transverse sections through the diencephalon (**11**) and rhombencephalon (**12**) of a *Xenopus* embryo at stage 33/34. Note the marginal postion of the cell bodies (arrowheads) and the rather dense plexus of NPY- immunoreactive fibers (arrows) at these early embryonic stages. Scalebar = 100 μm.

Brainstem

In the brainstem several immunoreactive cell groups occur. The most obvious group lies within the confines of the anteroventral tegmental nucleus. The cells are intensely stained and form a rather compact group (Fig. 7). In the midbrain, immunoreactive cell bodies were additionally found in tectal layers 4 and 6, the magnocellular nucleus of the torus semicircularis, and the interpeduncular nucleus. However, in these areas the cell bodies are only lightly or moderately stained and, except for the interpeduncular nucleus (Fig. 9), do not form distinct cell groups.

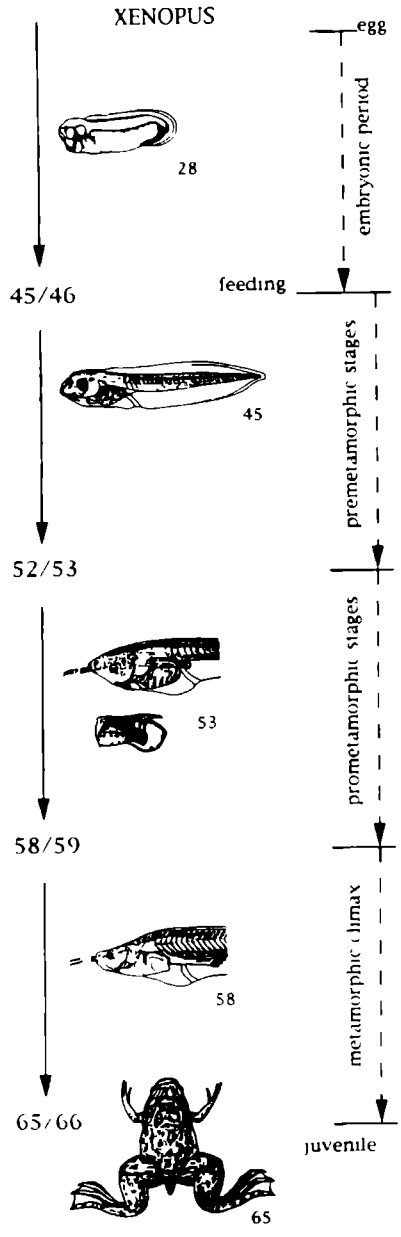


TABLE 1 Developmental stages of *Xenopus laevis* from egg to juvenile after Nieuwkoop and Faber (1967)

In more caudal parts of the brainstem, lightly and moderately stained cells were observed in the superior olivary nucleus, the solitary tract nucleus (Fig. 10), and, in a diffuse pattern, in the reticular formation. NPY immunoreactive fibers are widely spread throughout the brainstem. In the midbrain tectum they are particularly numerous in the deep tectal layers 2-6,8 and some sublaminae of layer 9. A very dense plexus of fibers is present in the ventral part of the interpeduncular nucleus and in a band-like region between the magnocellular nucleus of the torus semicircularis dorsally and the posterodorsal tegmental nucleus and isthmic nucleus ventrally (Fig. 9). Moderately dense plexuses occur in the raphe, the ventrolateral tegmentum, the lateral line area, and the solitary tract/area postrema complex (Fig. 10).

NPY immunoreactivity in the developing *Xenopus* brain

Developmental aspects of the distribution of NPY-immunoreactive cell bodies and fibers in *Xenopus* have been investigated in animals of which the age varied from early embryonic up to juvenile stages. For a detailed description of the development of *Xenopus* see Nieuwkoop and Faber (1967), but for clarity a few notes on the development will be made below (Table 1).

Two periods can be distinguished in the development, *i.e.* the embryonic and the larval period. The end of the embryonic period (stages 45/46) is marked by a total resorption of the external gills. The larval period, marked by independent feeding, is generally subdivided into three sets of stages: 1) *premetamorphosis* (stages 46-52), in which the tadpole merely grows in size and the buds of the hindlimbs appear on the lateral side of the body; 2) *prometamorphosis* (stages 53-58), characterized by the progressive formation of the hindlimbs. This period ends when the length of the tail is at its maximum and the more drastic changes of the metamorphosis start; and 3) *metamorphic climax* (stages 59-65), marking the period in which the transformation takes place from the tailed larva into the tailless, four-legged juvenile. On the basis of this subdivision, a detailed description of the distribution of NPY-immunoreactive cell bodies and fibers in the developing brain of *Xenopus* will be presented below.

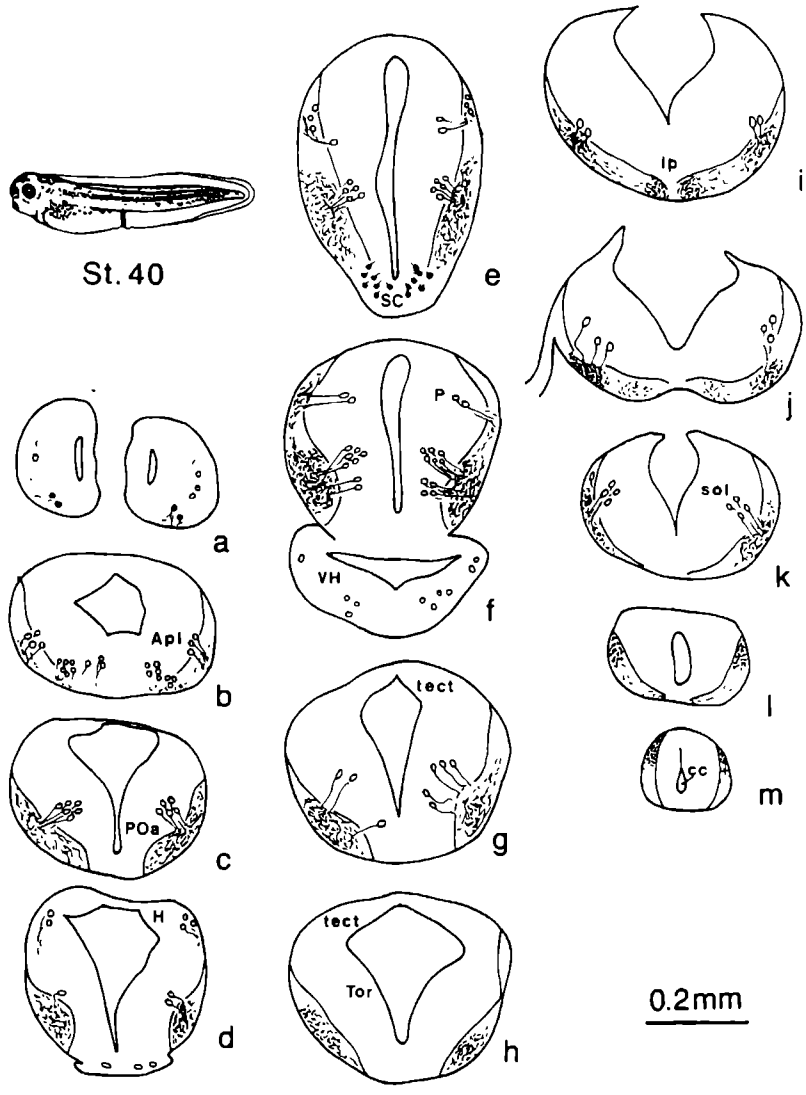
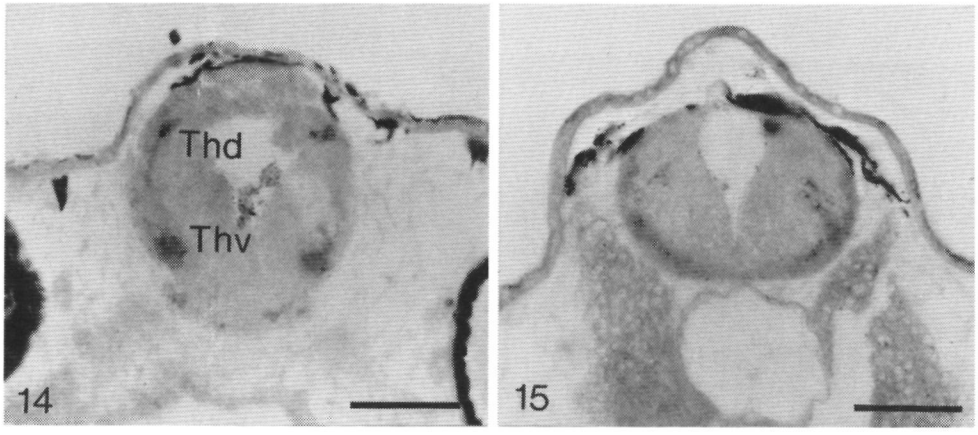


Fig. 13. Diagrams of transverse sections from rostral (a) to caudal (m) through the brain of a *Xenopus* embryo at stage 40 showing darkly (solid circles) and lightly (empty circles) stained cell bodies and fibers (curved lines, small dots). *Apl*, lateral amygdala; *cc*, central canal; *H*, habenula; *P*, posterior thalamic nucleus; *POa*, anterior preoptic nucleus; *SC*, suprachiasmatic nucleus; *sol*, solitary tract; *tect*, tectum mesencephali; Tor, torus semicircularis; VH, ventral hypothalamus nucleus

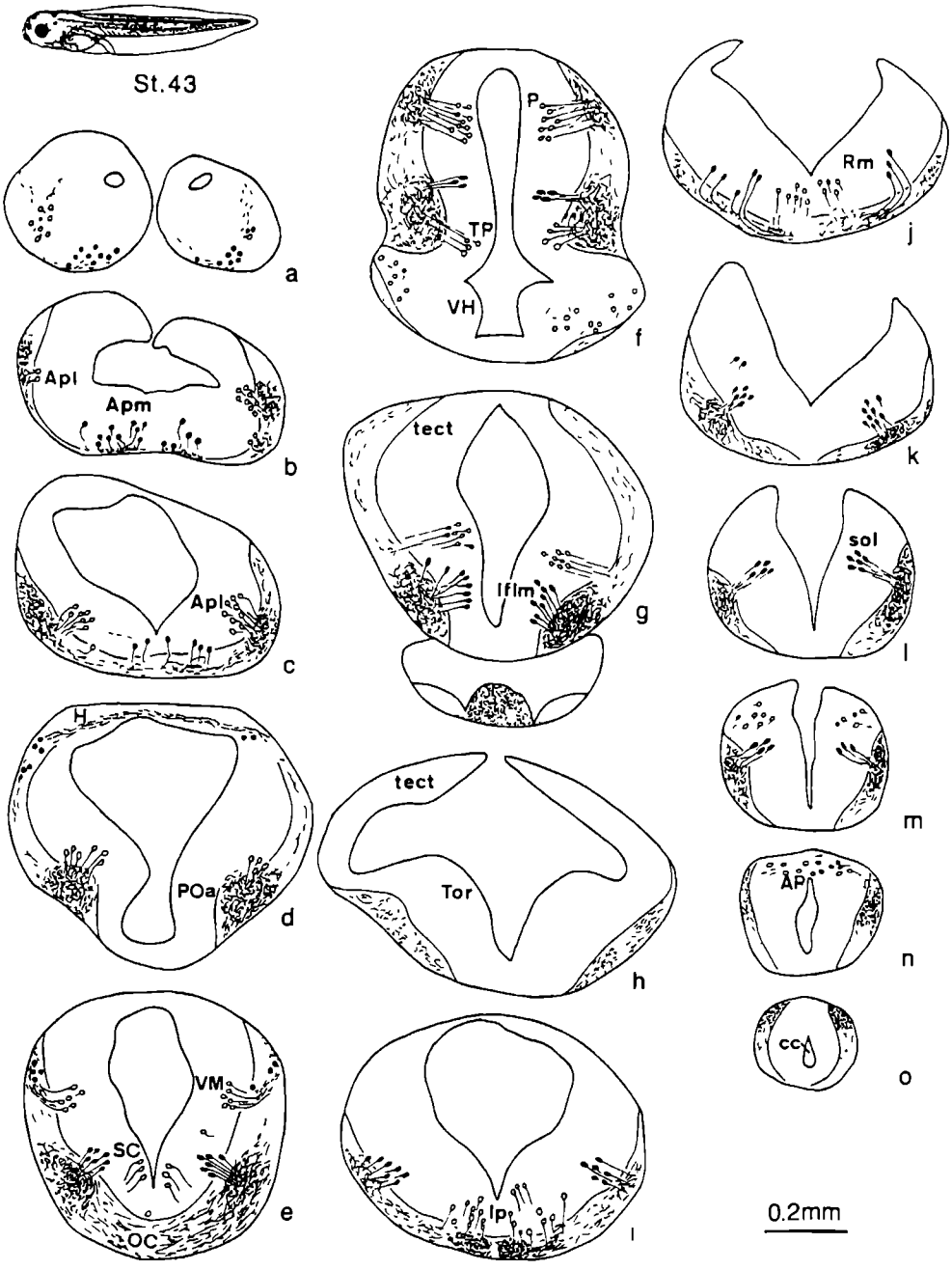
Early embryonic stages

Already at stages 33/34 a few, lightly stained cells are present in the developing neural tube. These cells are located in the diencephalic ventral thalamus and in the caudal rhombencephalon (Figs. 11,12). At these early stages, the immunoreactive cells occupy a marginal position within the wide cellular layer. The morphological appearance of these cells is always round and monopolar, with a principal process extending ventrolaterally into a narrow, fibrous zone. At these stages, immunoreactive fibers in this peripheral position were observed from rostral diencephalic levels to the obex.



Figs 14 and 15. Transverse sections through the diencephalon (**14**) and rhombencephalon (**15**) of a *Xenopus* embryo at stage 40. Note that three groups are visible in the diencephalon (dorsal thalamus, ventral thalamus and hypothalamus). The level of the section shown in **figure 14** is similar to that of **figure 13d**, whereas that of **figure 15** is the same as that of **figure 13k**. *Thd*, dorsal thalamus; *Thv*, ventral thalamus. Scalebar = 100 μm.

Fig. 16. Diagrams of transverse sections from rostral (a) to caudal (o) through the brain of a *Xenopus* embryo at stage 43 showing darkly (solid circles) and lightly (empty circles) stained cell bodies and fibers (curved lines, small dots). *AP*, area postrema; *Apl*, lateral amygdala; *Apm*, medial amygdala; *cc*, central canal; *H*, habenula; *Iflm*, interstitial nucleus of the medial longitudinal fascicle; *Ip*, interpeduncular nucleus; *P*, posterior thalamic nucleus; *POa*, anterior preoptic nucleus; *Rm*, medial reticular nucleus; *SC*, suprachiasmatic nucleus; *sol*, solitary tract; *tect*, tectum mesencephali; *Tor*, torus semicircularis; *TP*, posterior tubercle; *VH*, ventral hypothalamic nucleus; *VM*, ventromedial thalamic nucleus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At somewhat later embryonic stages (39-41), a substantial larger number of immunoreactive cells are found in the brain of *Xenopus* (Fig. 13). In the ventral part of the olfactory bulb a few, distinct immunoreactive cell bodies are observed (Fig. 13a) which merge caudally with a population of NPY-positive cells in the ventral part of the telencephalon (Fig. 13b). The latter population extends throughout the telencephalon. At caudal telencephalic levels, another group of lightly immunoreactive perikarya occurs in a more lateral position, *i.e.* in the developing amygdala (Fig. 13b). Also in the preoptic area, a cluster of lightly stained cells was found at these stages (Fig. 13c). Caudally, the latter cell group is continuous with immunoreactive cells that lie within the ventral thalamus and extends throughout the diencephalon (Figs. 13d-f,14).

Another distinct immunoreactive group of cells is observed in the dorsal thalamus. The most rostral cells lie close to the developing habenulae and are rather darkely stained (Fig. 14). Cells in the caudal portion of this group are located in the posterior thalamic area, just rostral to the tectum. They have long, arborized processes that enter the lateral marginal fiber layer (Figs 13d-f). In general, these cells are less darkly stained than those in the rostral portion of this group (Fig. 13f). Lightly stained cell bodies were identified immediately rostral to the chiasmatic ridge (Figs 13d,14). Caudally, immunoreactive cells in the suprachiasmatic region increase in number, as does the intensity of their staining (Fig. 13e). In the infundibular region, ventral and lateral to the infundibular recess, scattered, lightly stained cells occur (Fig. 13f). In the rostral part of the midbrain tegmentum and throughout the rhombencephalic tegmentum, lightly stained cells are present in a ventrolateral position (Figs 13g-m,15). Their processes run into the marginal fibrous layer which extends throughout almost the entire brain up to the dorsolateral margin of the spinal cord. In the retina, the first immunoreactive cells appear around these stages. Amacrine cells in the inner portion of the internal cell layer show moderate immunoreactivity and their incipient processes arborize within the scleral lamina of the inner plexiform layer.

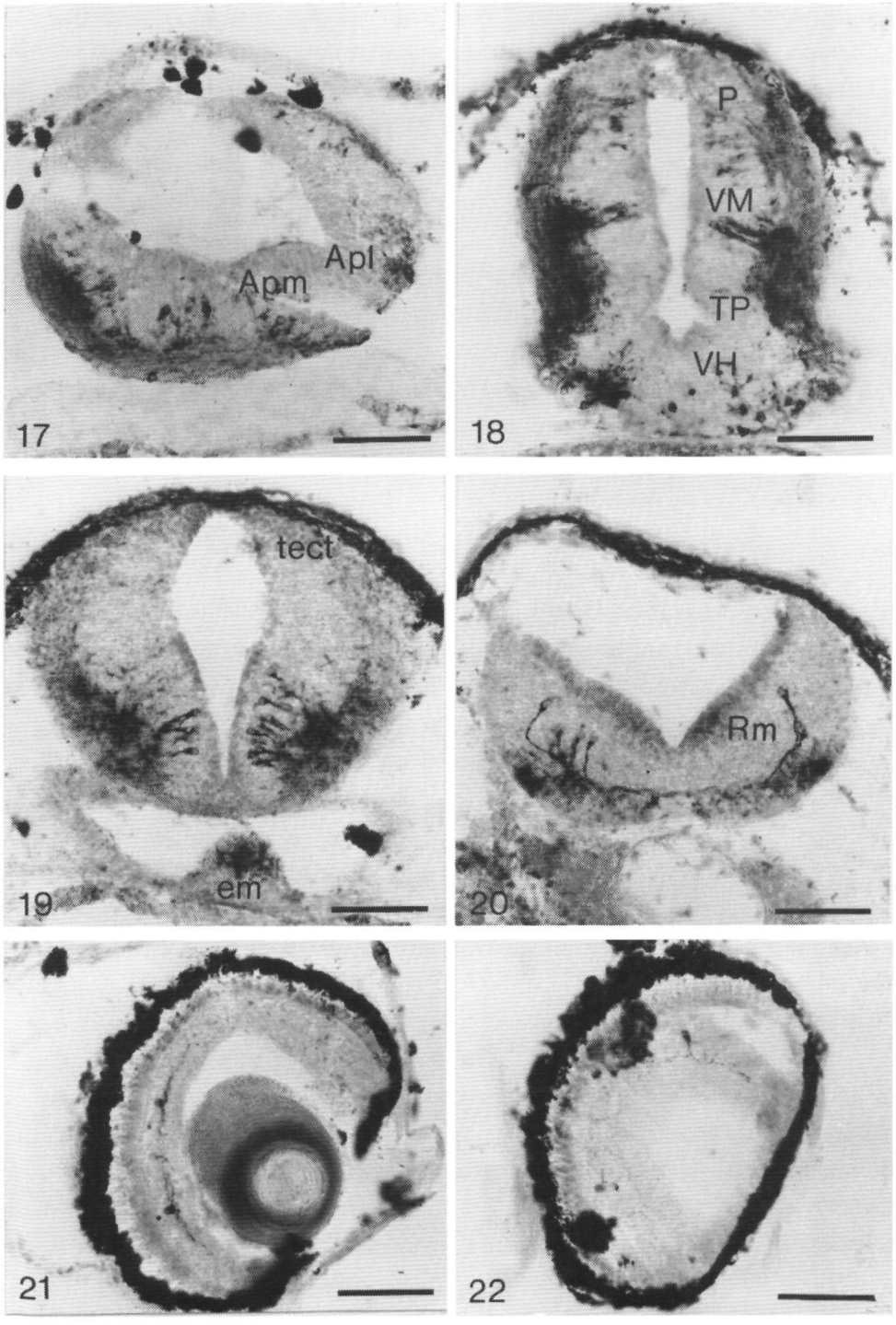
Late embryonic stages

Just before hatching (stages 43/44) an elaborated pattern of immunoreactive cell bodies and fibers is present (Fig. 16). The cell bodies are situated throughout the brain and their numbers are substantially larger than in previous stages. The

darkly stained cells in the ventral portion of the olfactory bulb have become laterodorsally accompanied by a second group of lightly stained cells (Fig. 16a). Throughout the telencephalon, up to the level of the amygdaloid region, the following general pattern is found (Figs 16b,c,17): (1) cells in a ventromedial position show a stronger immunoreactivity than those lying more dorsolaterally at the pallial/subpallial border; and (2) each cell group issues fibers to its own marginal fiber plexus. In the preoptic area a few, lightly stained cells are still present in a ventrolateral position and can be considered as the caudal extension of the lateral telencephalic NPY-positive cell group (Fig. 16d). At the same level, a new group of lightly stained cells appears adjacent to immunoreactive fibers in the habenular commissure (Fig. 16d). In the chiasmatic and more caudal diencephalic areas, numerous immunoreactive cell bodies are present that form rather distinct cell groups (Figs 16e, f, 18). In the suprachiasmatic region, a medial and a lateral group of cells can be distinguished (Fig. 16e). In the thalamus, distinct ventral and dorsal cell groups occur. The ventral group consists of small cells with processes that, rostrally, are directed dorsolaterally but caudally have a medial-to-lateral orientation (*cf.* Figs 16e and 16f). The dorsal cell group lies slightly caudal to the ventromedial thalamic group and contains cells that send their processes dorsolaterally forming a distinct plexus of immunoreactive fibers (Figs 16f,18). At caudal diencephalic levels, additional immunoreactive cell groups were observed in the posterior tubercle and the ventral hypothalamus (Fig. 16f,18). A distinct bundle runs from the hypothalamus to the neural and intermediate hypophyseal lobes.

In the rostral part of the midbrain, a population of large, round to oval-shaped cells appears in the tegmental area (Figs 16g,19). The cells form a band within the confines of the interstitial nucleus of the medial longitudinal fascicle as defined by Ten Donkelaar and De Boer-Van Huizen (1982). The processes of these cells arborize profusely in the ventrolateral part of the midbrain tegmentum.

Transverse sections through the caudal mesencephalon show a dense plexus of immunoreactive fibers in the superficial zone of the ventrolateral tegmentum, but no fibers are present in the developing tectum (Fig. 16h). More caudally, at the isthmic level, two groups of immunoreactive cell bodies were identified at these stages (Fig. 16i). One group occupies a dorsolateral position whereas the second consists of cells that lie ventromedially around the midline. A dense neuropile of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immunoreactive fibers is present ventral to the ventromedial group. Throughout the rhombencephalon numerous immunoreactive cell bodies were observed (Figs 16j-n, 20).

In the rostral and intermediate parts of the rhombencephalon, the cells in the lateral portion of the tegmentum show processes that first course into the direction of the marginal fiber layer and, upon reaching it, bend medially (Figs 16j,20). The cells in the medial part of the tegmentum are generally smaller and only slightly immunoreactive. In the caudal rhombencephalon, a distinct group of small, immunoreactive cells occurs in the area of the developing solitary tract (Figs 16l, 16m) whereas further caudally, at the level of the obex, an additional paired group of small, lightly stained cells appears dorsal to the solitary tract (Fig. 16m). From both sides of the brain these cell groups merge with each other dorsal to the central canal (Fig. 16n). In the retina, immunoreactive amacrine cells have strongly increased in number and in intensity of staining. The majority of their processes show well-elaborated arborizations within the scleral lamina of the inner plexiform layer (Fig. 21), but some cells display a distinct branching within the vitreal lamina of the inner plexiform layer (Fig. 22).

Larval period

Premetamorphic stages

By larval stages 48-50, the brain of *Xenopus* has undergone considerable maturation which is paralleled by a strong differentiation of the NPY system. The distribution of immunoreactive neuronal structures closely resembles the pattern observed in later stages during metamorphosis and in adults. Apparently, a substantial reduction in the number of cells has taken place around the time of

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Figs 17-22 Transverse sections of a *Xenopus* embryo at stage 43 showing NPY-immunoreactive cell bodies and fibers in the caudal telencephalon (**Fig. 17**, level as in **Fig. 16c**), the caudal diencephalon (**Fig. 18**, level as in **Fig. 16f**), the rostral midbrain (**Fig. 19**, level as in **Fig. 16g**) and in the rhombencephalon (**Fig. 20**, level as in **Fig. 16j**) **Figures 21** and **22** illustrate NPY immunoreactive amacrine cells and fibers in the retina at developmental stage 43 *Apl*, lateral amygdala, *Apm*, medial amygdala, *em*, median eminence; *P*, posterior thalamic nucleus; *Rm*, medial reticular nucleus, *tect*, tectum mesencephali; *TP*, posterior tubercle, *VH*, ventral hypothalamic nucleus, *VM*, ventromedial thalamic nucleus. Scalebar = 100 μm.

hatching (stage 47). Particularly in the olfactory bulb, the ventral telencephalon including the amygdala area and the lateral preoptic area, in the dorsal thalamus and in the brainstem, the number of imunoreactive cells is considerably lower. On the other hand, new groups of immunoreactive cells are found in the pallial areas, in the rostral part of the midbrain tectum and in the torus semicircularis. Immunoreactive fibers run from the rostral tectal pole to invade the tectal fiber layers. Furthermore, the plexus of immunoreactive fibers in the rhombencephalic dorsal lateral line has increased in density and the longitudinally coursing fibers in the ventrolateral tegmentum run to thoracic and lumbar spinal cord levels. Further development through *prometamorphic* and *metamorphic climax stages* is not marked by any significant change in the distribution of immunoreactive cell bodies and fibers throughout the brain.

DISCUSSION

The distribution of NPY-immunoreactive cell bodies and fibers has been studied in developing and adult brains of the South African clawed toad *Xenopus laevis*. We are particularly interested in studying the development of this neuropeptide, because it has been shown previously that NPY, together with dopamine (DA) and GABA, is involved in the neuroendocrine mechanism of background adaptation (Verburg-Van Kemenade et al., 1986a,b, 1987a; Jenks et al., 1993).

In their study of the distribution of NPY-immunoreactivity in the amphibian brain, Lázár et al. (1993) stated that their detailed description of NPY in the brain of *Rana esculenta* also holds for *Xenopus laevis* with two minor differences, namely the occurrence of NPY in the midbrain tectum and in the lower brainstem. However, our study of adult *Xenopus* material revealed several other differences as well. For example, a clear difference is observed in the distribution of NPY-immunoreactive fibers in the accessory olfactory bulb. Whereas an intense fiber staining is found in the glomerular layer of the accessory olfactory bulb in *Rana* (Lázár et al., 1993; own unpublished observations), the corresponding structure in *Xenopus* is devoid of immunoreactive fibers. Another difference concerns the distribution of NPY fibers in the octavo-lateral line area. In *Rana*, a dense plexus of NPY immunoreactive fibers is found in the saccular nucleus. In *Xenopus*, on the contrary, the saccular nucleus is devoid of immunoreactive fibers but the lateral

line area is rather densely innervated Lázar et al (1993) did not notice this difference because they considered the dorsal part of the alar plate in *Xenopus* as the saccular nucleus (their Figs 11D,E) However, in the latter species, contrary to the condition found in *Rana*, the lateral line system persists in adulthood and a tract-tracing study by Will et al (1985) has clearly shown that the area which is densely innervated by NPY fibers, receives primary lateral line afferent projections A similar, dense NPY innervation of the lateral line area has been described by Perroteau et al (1985) for the urodele, *Triturus cristatus*

The question arises as to the reason for the observed differences between our data and those of Lázár et al (1993) A possible explanation could be the use of colchicine by Lazár et al They report that colchicine-treated frogs (*Rana, Xenopus*) revealed a larger number and more intensely stained cells than untreated animals However, in the present study in which no colchicine was used, some areas of the *Xenopus* brain showed a clearly stronger immunoreactivity than described by Lázár et al For example, staining of perikarya in the suprachiasmatic nucleus, the ventromedial thalamic nucleus, the ventral hypothalamic nucleus and the anteroventral tegmental nucleus appears to be more intense in the present study It may, therefore, be concluded that the observed variation is not only due to the pretreatment with colchicine Possibly, some of the observed differences are related to the use of animals with a different physiological state, whereas others may be due to subtle differences in immunohistochemical procedures and the use of polyclonal antisera with different affinities for NPY The same arguments may be used to explain the differences observed in other vertebrate classes (for review, see Medina et al , 1992b)

Development of NPY-containing structures

The present study of the development of NPY-immunoreactive cell bodies and fibers has revealed that already at early embryonic stages immunoreactive cell bodies are present in the diencephalon and rhombencephalon In fact, the appearance of NPY-immunoreactive neuronal structures slightly preceeds that of catecholaminergic structures (Gonzalez et al , 1994) However, a remarkable difference is observed in the development of the two neurotransmitter systems The development of catecholamine systems is characterized by a steady increase in number of immunoreactive cells and fibers during embryonic and larval stages

The development of the NPY system also shows an increase in number of immunoreactive cells and fibers during the embryonic period, but after hatching, a substantial reduction in number of immunoreactive cells occurs, particularly in the basal forebrain and in the rhombencephalic tegmentum. At the same time, new cell groups express NPY-immunoreactivity, making it difficult to determine the exact time of appearance of immunoreactive cell groups as seen in adult brains. In early embryonic stages, therefore, we preferred to describe the immunoreactive cell groups on the basis of their position. Nevertheless, it may be assumed that the earliest observed cells in the ventral thalamus at stages 33/34 (Fig. 11) lie within the primordium of the ventromedial thalamic nucleus. Similarly, the three diencephalic cell groups observed in the dorsal thalamus, the ventral thalamus and in the hypothalamus (Fig. 14) at stages 39-41 may be considered the forerunners of the posterior thalamic, the ventromedial thalamic and the suprachiasmatic NPY cell groups of adults, respectively. The cells that form a band within the confines of the interstitial nucleus of the medial longitudinal fascicle (Fig. 16g) likely constitute the primordium of the NPY cell group in the anteroventral tegmental nucleus of adult *Xenopus*.

Development and background adaptation

Background adaptation in *Xenopus laevis* is enabled by movement of the pigment melanin in skin melanophores Melanin-filled melanophores are present from stage 33 onward but they do not react upon changing of the light intensity of the background. From stage 39, however, the control of melanophore pigment reaction is taken over by the pars intermedia of the pituitary gland. Upon release of α-MSH the pigment disperses, whereas a stop of α-MSH release leads to reaggregation of the pigment. This control is effectuated by neural factors that are believed to be produced in a number of brain centers. Involvement of the magnocellular nucleus, the suprachiasmatic nucleus and the locus coeruleus in the regulation of the melanotrope cells is suggested by tracing studies in which, as retrograde tracers, lypophylic dyes were applied to the hypophysis (Tuinhof et al., 1994a). Of these centers, the role of the suprachiasmatic nucleus has been studied in more detail. Neurons located in the suprachiasmatic nucleus likely receive a direct retinal input (Tuinhof et al., 1994a). They also show an increase in the production of the α-MSH release-inhibiting neurotransmitter NPY after toads are

placed on a white background (Tuinhof et al., 1993b). A few of these suprachiasmatic NPY- immunoreactive cells probably contain also dopamine, as they are tyrosine hydroxylase-immunoreactive as well (Tuinhof et al., 1994b). This coexistence of NPY and DA is in line with the previously demonstrated colocalisation of these two neurotransmitters in the terminal network of the pars intermedia (de Rijk et al., 1990a, 1992)

The present study shows that NPY becomes detectable in the suprachiasmatic nucleus at stage 39, which coincides with (1) the differentiation of the adenohypophyseal primordium into the distal and intermediate hypophysis (Nyholm, 1977; Nyholm and Doerr-Schott, 1977; Eagleson et al., 1986; Verburg-Van Kemenade et al., 1984); (2) the start of melanophore sensitivity for α-MSH (Verburg-Van Kemenade et al., 1984); (3) the start of the production of α-MSH in the pars intermedia (Nyholm and Doerr-Schott, 1977); (4) the appearance of DA-innervation of the pars intermedia of the hypophysis (Terlou and Van Straaten, 1973); (5) the first sign of sensitivity of the melanotropes for dopamine (apomorphine); and (6) the earliest reaction of the melanotropes to placing the animal on a changed background. This coincidence strongly suggests that the suprachiasmatic nucleus and, in particular, the presence of NPY and DA in this nucleus play a key role in the (inhibitory) control of melanotrope cell activity during the process of background adaptation.

ACKNOWLEDGMENTS

The authors are greatly indebted to Dr H. Vaudry (Rouen, France) and Dr J.D. Mikkelsen (Copenhagen, Denmark) for the gifts of anti-NPY sera, and to Mr D. de Jong for preparing the photomicrographs. This study was supported by EC HCM grant ERBCHRXCT920017 and an INSERM/NWO travel grant, both to EWR, Spanish Research Grant DGICYT PB 90-0628 to AG, and NATO Collaborative Research Grant CRG 910970 to WJAJS and AG.

CHAPTER 5

Immunocytochemistry and *in situ* hybridization of neuropeptide Y in the hypothalamus of *Xenopus laevis* in relation to background adaptation

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in Neuroscience ***51****; 667-675 (1993)*

ABSTRACT

The amphibian *Xenopus laevis* is able to adapt to a dark background by releasing melanophore-stimulating hormone from the pars intermedia of the hypophysis. The inhibition of melanophore stimulating hormone release is accomplished by neuropeptide Y-containing axons innervating the pars intermedia. To determine the production site of neuropeptide Y involved in this inhibitory control, the distribution of neuropeptide Y in the brain has been investigated by immunocytochemistry and *in situ* hybridization. Immunoreactive cell bodies were visualized in, among others, the ventromedial and posterior thalamic nuclei, and the suprachiasmatic and ventral infundibular hypothalamic nuclei. A positive hybridization signal with a *Xenopus*-specific probe for pre-proneuropeptide Y-mRNA was found in the diencephalic ventromedial thalamic nucleus and in the suprachiasmatic nucleus. With both immunocytochemistry and *in situ* hybridization, suprachiasmatic neurons appeared to be stained only in animals adapted to a white background; animals adapted to a black background showed no staining. Quantitative image analysis revealed that this effect of background adaptation is specific for suprachiasmatic neurons because no effect could be demonstrated of the background light condition on the ventral infundibular nucleus (immunocytochemistry) or the ventromedial thalamic nucleus (*in situ* hybridization).

These results indicate that neurons in the suprachiasmatic nucleus enable the adaptation of *X. laevis* to a white background, by producing and releasing neuropeptide Y that inhibits the release of melanophore-stimulating hormone from the melanotrope cells in the pars intermedia of the hypophysis.

INTRODUCTION

The complex neuroendocrine mechanism that enables the South African clawed toad *Xenopus laevis* to adapt physiologically to the light intensity of the background by changing the colour of the skin, has been extensively investigated. A major part of the research has been focused on the functioning of the melanotrope cells in the neurointermediate lobe of the hypophysis (Jenks and Van Zoest, 1990; Roubos, 1992). These cells release melanophore-stimulating hormone (α-MSH), which causes the dispersion of melanin in the skin

melanophores. *In vitro* superfusion studies with intact neurointermediate lobes and with single melanotrope cells have indicated that the release of α-MSH is inhibited by three neurochemical messengers, dopamine (DA), γ-amino butyric acid (GABA) and neuropeptide Y (NPY; De Koning et al., 1991; Verburg-Van Kemenade et al., 1986a,b;1987a). All three messengers have been shown to co-exist in fibers that make synaptic contacts with the melanotrope cells (De Rijk et al., 1990a; 1992; Van Strien et al., 1991). These data favour the hypothesis that *Xenopus* neurons that produce DA, NPY and GABA are involved in the process of skin bleaching during adaptation to a white background, by inhibiting α-MSH release from the melanotrope cells. This hypothesis is central in the present study.

In *Xenopus laevis* tadpoles, the occurrence of DA has been studied with the formaldehyde-induced fluorescence method (Goos, 1969; Terlou and Ploemacher, 1973; Terlou and Van Kooten, 1974). Fluorescent neurons are present in various parts of the brain, including the anterior preoptic nucleus and the hypothalamic paraventricular organ, dorsal infundibular nucleus and posterior tubercle. Of these centers, neurons in the caudal part of the paraventricular organ were thought to be involved in the control of background adaptation, because they seem to project to the pars intermedia (Goos, 1969; Terlou and Ploemacher, 1973). Recent immunocytochemical studies on adult *Xenopus*, however, showed that the neurons of the paraventricular organ react with anti-DA but not with anti-tyrosine hydroxylase (anti-TH), whereas neurons in the suprachiasmatic nucleus are immunoreactive to both antisera (González et al., 1993; Tuinhof et al., 1993a). As the latter cells also send DA- and TH-immunoreactive axons to the hypophysis, the suprachiasmatic nucleus may be involved in the control of the melanotrope cells.

Since DA and NPY co-exist in the nerve fibers innervating the pars intermedia, a next step in the identification of the neuronal somata that control melanotrope cell activity is the study of the distribution of NPY in the *Xenopus* brain. NPY has been reported to occur in the preoptic region (Verburg-Van Kemenade et al., 1987a) but until now no detailed localization studies have been performed.

In the present study, the occurrence of NPY has been examined with immunocytochemistry using an anti-NPY serum, and with *in situ* hybridization using a probe for preproNPY-mRNA made from our cDNA hypothalamic

library (Van Riel et al., 1993). In order to correlate the activity of NPY-producing brain centers with the process of background adaptation, brain sections of animals adapted to either a white or a black background were investigated with immunocytochemistry, *in situ* hybridization and quantitative image analysis.

EXPERIMENTAL PROCEDURES

Animals

Adult (aged eight months) specimens of *Xenopus laevis*, with a body weight of 28-32 g, were reared in our laboratory. Full background adaptation was achieved by keeping the animals for 3 weeks on a white or a black background. They were kept in water of 22 °C at constant illumination and fed trout pellets (Trouvit; Trouw, Putten, The Netherlands). Before dissection the animals were anaesthetized by immersion in a solution of 0.1% tricaine methane sulfonate (MS 222; Sandoz, Switzerland).

Immunocytochemistry

For immunocytochemistry, toads were transcardially perfused with cold 0.6% sodium chloride solution, carbogene-aerated to pH 6.9, for 5 min, followed by perfusion fixation in Bouin's fluid, for 15 min. After dissection, the brains were postfixed in the same fixative, for 3 h. Paraffin embedded, serial vibratome cut, horizontal and transversal sections (8 µm thick) of the forebrain and midbrain were mounted on gelatine-coated glass slides, air-dried, deparaffinized and rehydrated. The subsequent staining procedure (for details see, De Rijk et al., 1990) was carried out at 20 °C, unless stated otherwise. In short, sections were pretreated with 50 mM Tris-buffered saline (pH 7.6) containing 150 mM NaCl and 0.3% Triton (TBS-TX; Sigma, St Louis, MO, U.S.A.). Then, they were incubated in 20% normal goat serum (NGS) preventing aspecific staining, for 30 min, and with rabbit-anti-NPY (1:2000) at 4 °C for 18 h. Second antiserum was goat anti-rabbit IgG (1:50; Nordic; Tilburg; The Netherlands), applied for 90 min. Finally, incubation was performed with rabbit peroxidase-antiperoxidase (PAP; 1:1000; Nordic), for 90 min. All incubations and rinses were carried out

in TBS-TX. The high specificity of the anti-NPY serum has been demonstrated previously (Danger et al., 1985). Immunobinding was visualized by rinsing the sections in Tris-HCl (pH 7.6) and subsequent incubation in 0.04% 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma), 0.25% nickel ammonium sulfate and 0.015% H_2O_2 in 50 mM Tris-HCl (pH 7.6), for 40 min. The reaction was terminated by several rinses in Tris-HCl. Finally, slides were dehydrated and embedded after or without counterstaining in hemalum.

In situ *hybridization*

For *in situ* hybridization, freshly dissected brains were fixed by immersion in Bouin's fluid using diethyl pyrocarbonate water as a solvent. This water was used throughout the subsequent procedure. After deparaffination and dehydration, sections were pretreated with 0.1% pepsin (p-7000; Sigma) in 0.2 N HCl, at 37 °C for 15 min. Sections were postfixed in 2% paraformaldehyde (pH 7.3) for 4 min. Then they were dehydrated for 10 min in 100% alcohol and air-dried. As a probe, a *Xenopus*-specific antisense preproNPY-mRNA probe made from our *Xenopus* hypothalamic cDNA library (Van Riel et al., 1993), was used. The probe was labeled with digoxigenin using a digoxigenin labeling kit according to the manufacturer's instructions (Boehringer, Mannheim, Germany). The hybridization mixture contained 50% deionized formamide, 4 x SSC (1 x SSC = 0.15 M NaCl and 0.015 M sodium citrate), 10% dextran, 0.1% polyvinyl pyrrolidone, 0.1% bovine serum albumin, 0.1% Ficoll 400 (Pharmacia, Uppsala, Sweden), 200 μg/ml yeast tRNA (Boehringer) and 25 mM sodium phosphate buffer (pH 7.0). This mixture was heated at 70 °C for 5 min, before applying to the sections. Per slide 1 µl of the probe (300 ng), dissolved in 150 µl hybridization mixture, was applied. Subsequently, sections were covered with RNAse-free coverslips. Hybridization was performed in a moist chamber, at 50 °C for 16 h. After hybridization, slides were rinsed twice with 2 x SSC for 30 min at 20 °C, once in 1 x SSC for 30 min at 20 °C, once in 0.5 x SSC for 30 min at 20 °C, and finally, once in 0.5 X SSC for 30 min at 37 °C. After rinsing in sodium phosphate-buffered saline (PBS; pH 7.4) the sections were incubated for 30 min in 2% NGS, 0.3% PBS-TX and 0.1% bovine serum albumin in PBS, followed by 2 h in anti-dioxigenin conjugate (1:500; Boehringer), 0.1% NGS and 0.3% TX in PBS. After rinsing in PBS (2 x 15 min) and in a buffer

containing 10 ml 0 1 M Tris, 0 1 M NaCl and 0 05 M $MgCl_2$ (pH 9 5), for 2 min, incubation was performed with the substrate, namely 45 μl nitroblue tetrazolium (Boehringer) and 35 μl X-phosphate (4-toluidine salt, Boehringer) in the buffer, at 4°C for 16 h Finally, sections were rinsed and embedded with glycerin in 10 mM PBS (1 10)

Image analysis

To determine possible changes in size and in degree of immunoreactivity of NPY-positive neurons as a function of background adaptation, sections through the brains of white- and black-adapted animals were studied with an automated image analysis system (VIDAS, Kontron, Munchen, Germany) Per animal, measurements were carried out on all profiles of immunoreactive perikarya present in a median section through the paired brain nucleus under investigation (suprachiasmatic nucleus, ventral infundibular nucleus) Per cell profile in the section, the mean cell profile area (expressed in μm^2) and the staining intensity (optical density) were determined Optical density was expressed in grey level values, with value 0 is white (full light transmission) and value 255 is black (full light absorption) For *in situ* hybridization, the same procedure was followed, but measurements were carried out on the suprachiasmatic nucleus and the ventromedial nucleus The data were entered into a one-way analysis of variance (α=5%, Bliss, 1967), which was preceded by tests for the homogeneity of variance (Steel and Torrie, 1960) and the joint assessment of normality (Shapiro and Wilk, 1965)

RESULTS

Immunocytochemistry

General occurrence of neuropeptide Y-positive neurons

NPY-positive perikarya are dispersed throughout the central nervous system of *X laevis* In the telencephalon, NPY-positive cell bodies occur in the olfactory bulb, in the dorsal, medial and lateral pallium, in the medial and lateral septum, in the striatum, in the nucleus accumbens and in the medial part

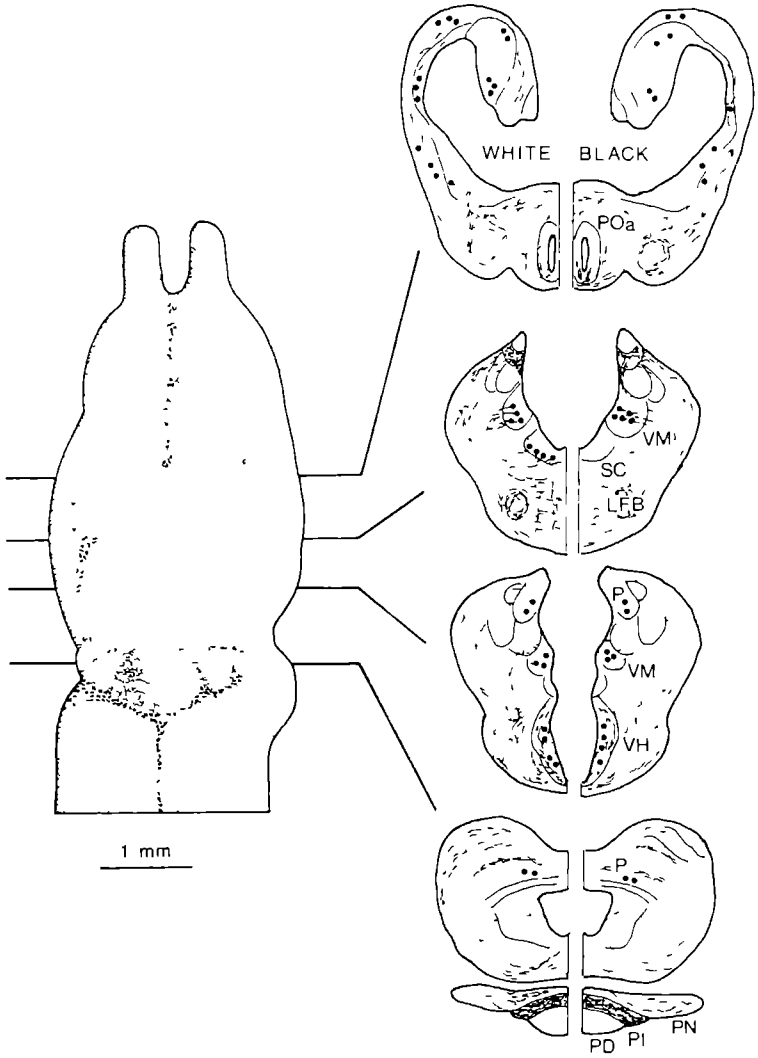
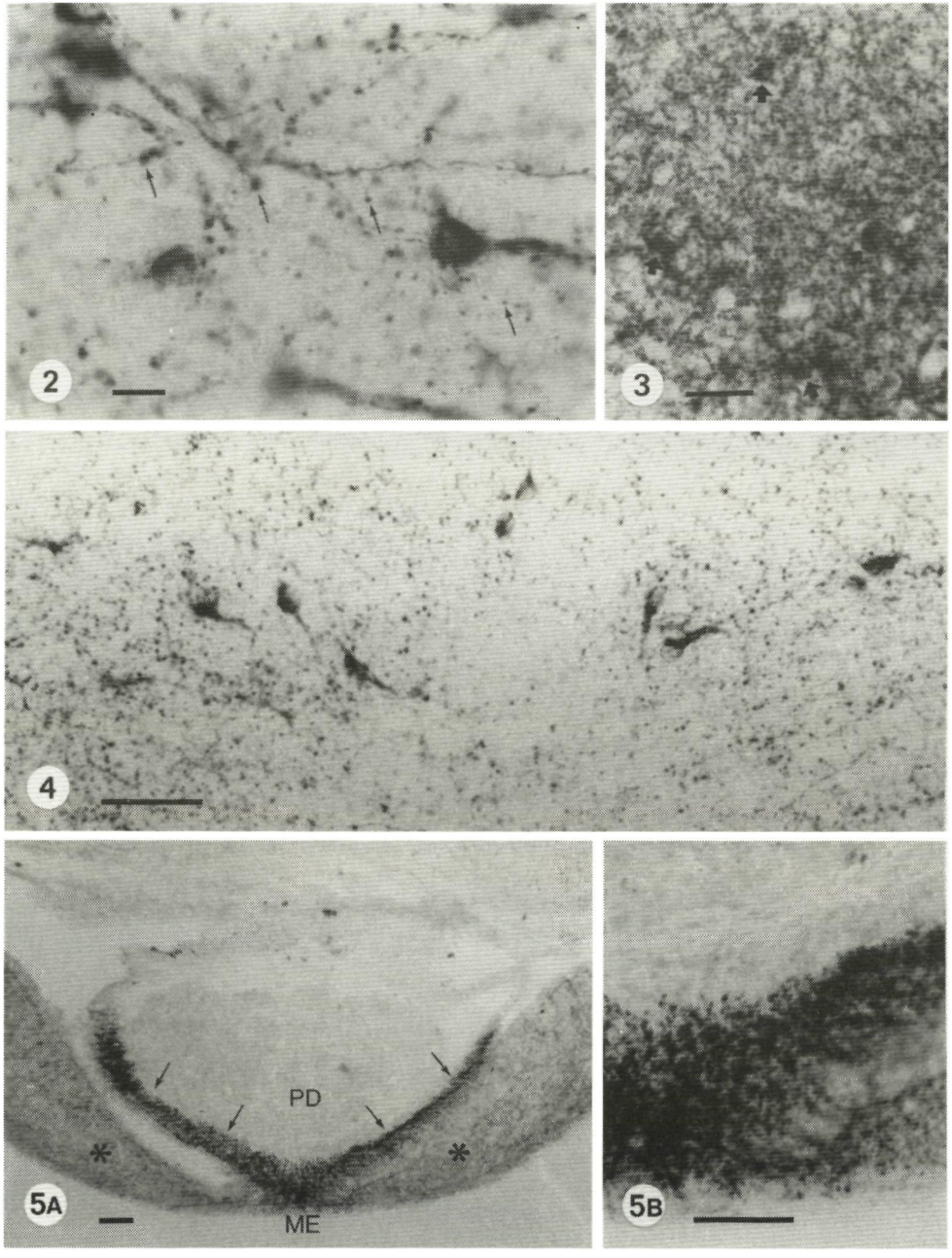


Fig 1. Distribution of NPY-positive neurons in the brain of *X. laevis* At four transverse levels (see ventral view of the brain, at the left), immunoreactive cell bodies (large dots) and fibers (small dots and wavy lines) have been indicated in schematic drawings (at the right) Each drawing consists of two halfs, the left one representing the situation in animals adapted to a white background, the right one that in black-adapted animals Note absence of positive neurons in suprachiasmatic nucleus (SC) in the latter situation *LFB*, lateral forebrain bundle, *P*, posterior thalamic nucleus, *PD*, pars distalis of the hypophysis, *PI*, pars intermedia of the hypophysis, *PN*, pars nervosa of the hypophysis, *POa*, anterior preoptic nucleus, *VH*, ventral infundibular nucleus, *VM*, ventromedial thalamic nucleus The nomenclature has been adopted from Neary and Northcutt 1983, González et al , 1993


2
3
4
PD
ME
5A
5B

of the amygdala (Fig. 1). The majority of NPY-positive neurons is located in the diencephalon, namely in the thalamic ventromedial (Fig. 2) and posterior nuclei, and in the hypothalamus (see below). In the mesencephalon some NPY-positive neurons are present, namely in the anteroventral tegmental nucleus.

NPY-positive fibers with a beaded appearance are diffusely present throughout the central nervous system. Distinct fibre plexuses are seen in the outer half of the mitral layer of the olfactory bulb with exception of the midline area, in the striatum, in the diagonal band of Broca and in the amygdala. Such dense fibre labeling is also present in the hypothalamus (see below).

The hypothalamus

In the hypothalamus NPY-positive cell bodies are present in the ventral infundibular nucleus and in the suprachiasmatic nucleus. Fibre plexuses occur in the lateral forebrain bundle, in the anterior preoptic nucleus, in the ventral infundibular nucleus, in the ventral part of the infundibulum and in the internal layer of the median eminence (Fig. 1).

Each of the paired ventral infundibular nuclei contains about 40-60 NPY-positive cell bodies (Fig. 3). They measure between 15-20 μm in length and 10-15 μm in width. Their axons run into various directions. Each of the paired suprachiasmatic nuclei contains 30-50 immunoreactive cell bodies (Fig. 4). They have dimensions similar to the NPY-positive cells in the ventral infundibular nuclei. Their axons run into caudoventral direction before becoming indistinct from other fibers coursing in the same area. From this area, many fibers run via the median eminence (Fig. 5) to the neural lobe of the hypophysis. Here they ramify before penetrating the pars intermedia, where they form a strongly immunoreactive fibre plexus around the melanotrope cells (Fig. 6).

Figs 2-5. Transverse section through ventromedial thalamic nucleus, with various NPY-immunoreactive neurons. Arrows indicate axonal beads Scalebar = 10 μm. **Fig. 3** NPY-positive parikarya (arrows) amidst numerous strongly staining axons in ventral infundibular nucleus. Scalebar = 25 μm **Fig. 4** NPY-positive neurons in left and right suprachiasmatic nuclei of animal adapted to a white background Scalebar = 50 μm **Fig. 5** Ventral infundibular area (asterisks) with (A) NPY-positive axons coursing to the hypophysis (arrows) *ME*, median eminence, *PD*, pars distalis of the hypophysis Scalebar = 10 μm (B) Detail of axons entering hypophysis Scalebar = 50 μm

All NPY-positive neurons mentioned above were clearly visible irrespective the state of background adaptation of the animal (either white or black), with exception of the neurons in the suprachiasmatic nucleus. The latter neurons were found to be immunopositive only in animals that had been kept on a white background (Fig. 7).

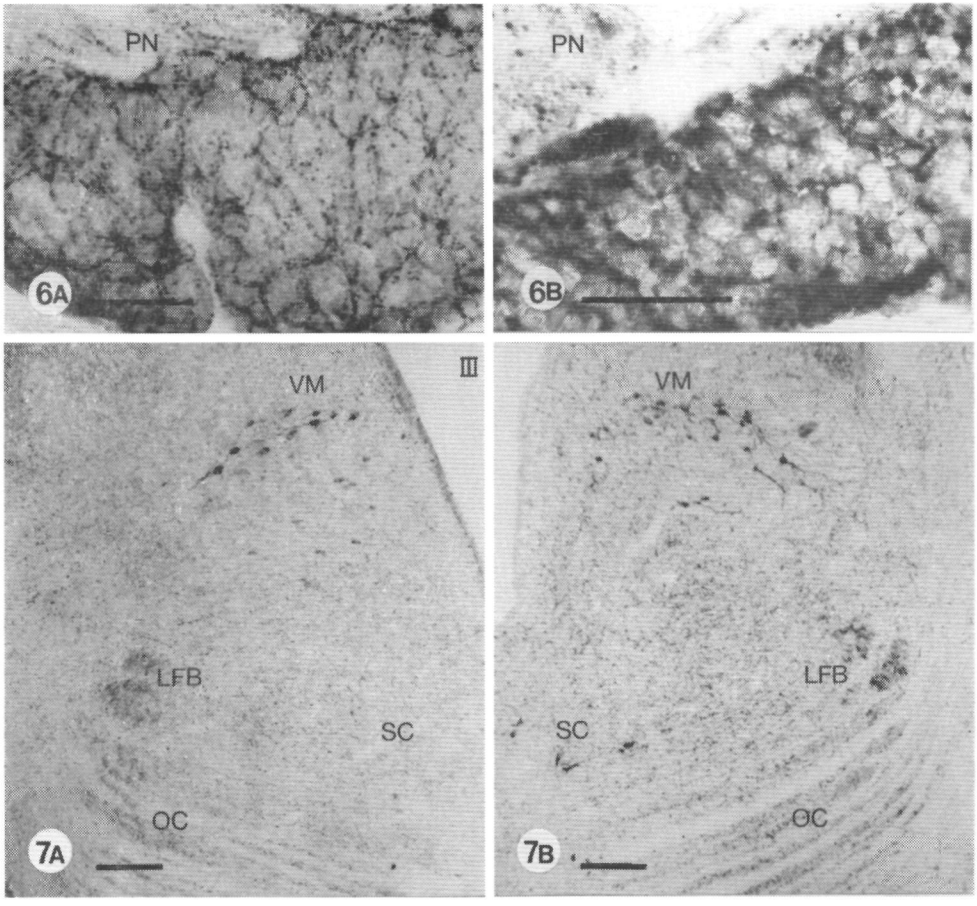
Quantitative image analysis reveals that NPY-positive neurons in the suprachiasmatic nucleus of white-adapted animals show a strong staining intensity, with a mean optical density of about 140 (Table 1). Since in black-adapted animals, sections of the suprachiasmatic nucleus do not show NPY-positive neurons, the NPY-producing cells could not be identified beyond doubt. In order to obtain a quantitative impression of the amount of background optical density in these cells, measurements were performed, in these sections on unstained suprachiasmatic neurons. This procedure shows a highly significant ($P < 0.001$) difference (about 30 optical density units) in staining intensity between white- and black-adapted animals. In order to determine whether background adaptation specifically affects the NPY-producing cells in the suprachiasmatic nucleus, image analysis was extended to the other NPY-positive centre in the hypothalamus, the ventral infundibular nucleus. No differences were found for the NPY-positive neurons in this nucleus between white- and black-adapted animals, either with respect to the mean optical density of the cytoplasm or to the mean cell profile area (Table 1).

Compared to the pars intermedia of white-adapted animals, that of black-adapted toads is remarkably large (Fig. 6), due to the presence of much larger melanotrope cells. In white-adapted animals the NPY-positive fibers form a very dense, strongly stained plexus, whereas the plexus in black-adapted ones is only moderately stained (Fig. 6B).

In situ *hybridization and background adaptation*

In brains of white- and black-adapted animals, perikarya hybridizing with the RNA-probe for preproNPY were seen in the ventromedial thalamic nucleus (Figs 8,9A). Furthermore, hybridizing neurons were only found in the hypothalamus, namely in the suprachiasmatic nucleus (Figs 8,9B). These

neurons showed a positive signal in white-adapted animals and revealed the same location and morphology as the suprachiasmatic cells staining with anti-NPY. In the hypothalamus of black-adapted animals, including the suprachiasmatic nucleus, no hybridization signals were observed.



Figs 6 and **7**. NPY-positive axons forming a plexus around the immunonegative melanotrope cells in the pars intermedia. Adaptation to (A) black or (B) white background. *PN*, pars nervosa. Scalebar = 50 µm. **Fig. 7**. Survey of transverse section through the midbrain of *X. laevis*, with NPY-positive lateral forebrain bundle (LFB) and ventromedial nuclei (VM). NPY-immunoreactive neurons in the suprachiasmatic nucleus (SC) are present in animals adapted to a white background (B) but absent in black-adapted ones (A). *III*, third ventricle; *OC*, optic chiasm. Scalebar = 100 µm.

Image analysis reveals that suprachiasmatic neurons of white-adapted animals have a hybridizing signal with an optical density between 160 and 170 (Table 2). Non-hybridizing cell bodies in the suprachiasmatic nucleus of black-adapted animals show a much lower density of 120 ($P < 0.001$). In order to determine whether background adaptation specifically influences the expression of preproNPY mRNA in suprachiasmatic neurons, the other hybridizing brain centre, the ventromedial thalamic nucleus, was studied as well. In contrast to the suprachiasmatic neurons, the ventromedial cells did not show any difference in the mean surface area of the perikaryon profile (Table 2) or in the strength of the hybridizing signal, when white- and black-adapted animals were compared.

DISCUSSION

Neurons produce neuropeptide Y

In the present *in situ* hybridization experiment a preproNPY-probe was used which we recently derived from a *Xenopus* hypothalamic cDNA library (Van Riel et al., 1993). It is shown that in *X. laevis* neurons in the suprachiasmatic nucleus and the ventromedial thalamic nucleus produce preproNPY mRNA. Therefore, it can be concluded that these neurons produce NPY. This notion is supported by the immunocytochemical studies, which demonstrated that neurons in both nuclei can be immunologically stained using an anti-NPY serum.

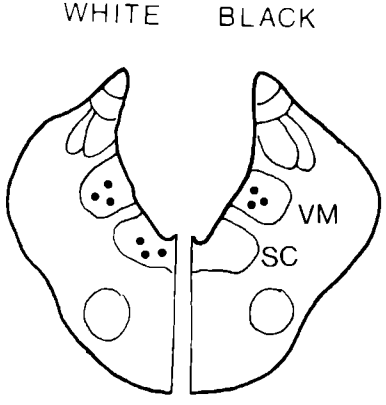


Fig. 8. Schematic representation of *in situ* hybridization with RNA probe for preproNPY, of transverse section through diencephalon of *X. laevis* Hybridizing neurons occur in the ventromedial nucleus (VM) of both white- (left half drawing) and black-adapted animals (right half). Suprachiasmatic neurons (SC) only hybridize in white-adapted toads

NPY-immunoreactivity also appears to be present in various other neurons in the central nervous system which, however, do not show a positive hybridization signal. This discrepancy might be explained by assuming that the immunoreactivity of these neurons is due to cross-reaction of the serum, for instance with peptides that are not related to NPY but share a common epitope with this peptide. This explanation does not seem to be likely, however, for the following two reasons: (i) the anti-NPY serum has a high specificity and does not cross-react with a wide variety of other neuropeptides (Danger et al., 1985), and (ii) in the related amphibian species *Rana ridibunda*, NPY has been demonstrated to be present at similar sites in the central nervous system as in *X. laevis*, namely in telencephalic, thalamic, preoptic, infundibular and mesencephalic areas and in the medulla oblongata, not only with immunocytochemistry but also with high performance liquid chromatography (Danger et al., 1985). Therefore, it seems likely that in these neurons the amount of preproNPY-mRNA is too low to be detected with the hybridization method.

Suprachiasmatic neurons control the pars intermedia

In order to find the origin of the neuronal center(s) that control the activity of the melanotrope cells in the pars intermedia, we focused on NPY-immunoreactive neurons in the hypothalamus, namely in the suprachiasmatic nucleus and the ventral infundibular nucleus. Of these, anti-NPY-positive suprachiasmatic neurons send their axons in caudoventral direction, before becoming indistinct in the infundibular area. From this area many fibers run via the median eminence to the pars intermedia, where they form a strongly NPY-immunoreactive fiber plexus around the melanotrope cell. This observation is in line with the hypothesis that neurons in the suprachiasmatic nucleus contact the melanotropes (Tuinhof et al., 1993a). It is, moreover, strongly supported by the observation that the suprachiasmatic neurons are only stained with the NPY-serum in animals that have been adapted to a white background, a situation in which inhibitory mechanisms acting on the melanotrope cells would be expected to be active. The other NPY-positive centre in the hypothalamus, the ventral infundibular nucleus, does not show a clear projection toward the infundibular area.

Table 1. Quantitative image analysis of anti-neuropeptide Y-immunoreactive neurons in the suprachiasmatic and ventral infundibular nuclei in *Xenopus laevis* adapted to either white or black background

	Optical density units	Cell profile in μm^2
SC white	142 ± 4^a	
SC black	112 ± 1^b	
VH white	158 ± 3^a	15.8 ± 0.9^c
VH black	153 ± 6^a	14.6 ± 0.8^c

The degree of NPY-immunoreactivity has been expressed in optical density units, the cell size as μm^2 of cell profile area (mean ± standard errors of the mean; $n = 6$). Groups sharing the same superscript do not differ significantly ($P < 0.001$).

Moreover, image analysis does not demonstrate any effect of the state of background adaptation on the appearance (size and the degree of NPY-immunoreactivity) of these neurons.

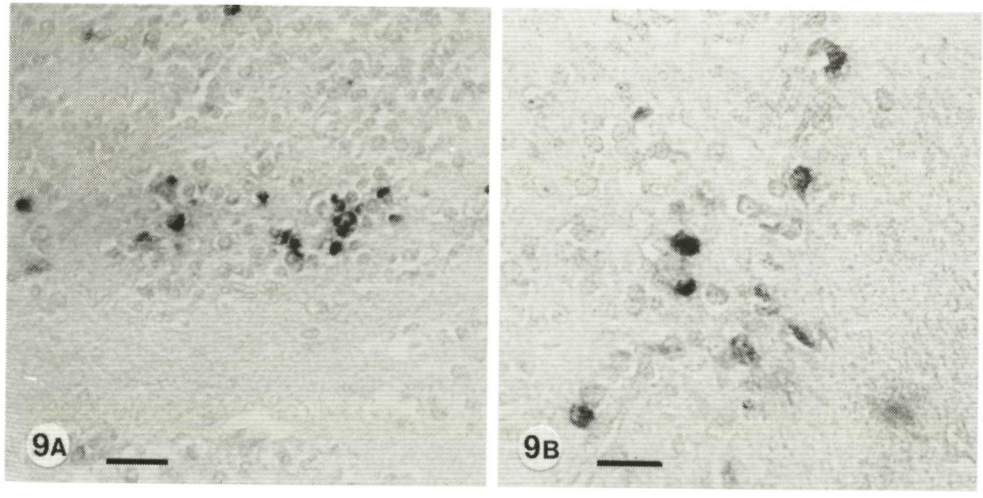


Fig. 9. *In situ* hybridization with RNA probe for preproNPY, showing hybridizing cell bodies in sagittal section of (**11A**) ventromedial nucleus of animal adapted to a black background (scalebar = 200 μm) and of (**11B**) suprachiasmatic nucleus of animal adapted to a white background (scalebar = 100 μm).

Therefore, one would assume that the background light condition specifically affects the activity of the suprachiasmatic nucleus. This assumption is strongly supported by the *in situ* hybridization experiment showing a positive hybridization signal in suprachiasmatic neurons only in animals adapted to a white background; no signal was found in black-adapted toads. Apparently, in white-adapted animals these neurons express the NPY-gene to a high level, whereas NPY production is low under conditions of black-adaptation. Again, the specificity of this effect of background adaptation on suprachiasmatic neurons is apparent, because no effect was found on the strength of the hybridization signal in the only other hybridization-positive centre found in the brain, the ventromedial thalamic nucleus.

Table 2 Quantitative image analysis of neurons in the suprachiasmatic and ventromedial thalamic nuclei showing a positive *in situ* hybridization signal with preproneuropeptide Y probe, in *Xenopus laevis* adapted to either white or black background

	Optical density units	Cell profile in μm^2
SC white	164 ± 3^a	
SC black	122 ± 1^b	
VH white	163 ± 4^a	21 ± 3^c
VH black	163 ± 0.3^a	24 ± 2^c

The amount of preproNPY-mRNA has been expressed in optical density units, the cell size as μm^2 of cell profile area (mean ± standard errors of the mean, $n = 4$) Groups sharing the same superscript do not differ significantly ($P < 0\ 001$).

In vivo *action of suprachiasmatic neurons*

In vitro superfusion studies have shown that NPY has an inhibitory effect on the release of α-MSH from the melanotrope cells of *X. laevis* (Verburg-Van Kemenade et al., 1987a). Therefore, we propose that in animals kept on a white background, neurons in the suprachiasmatic nucleus produce, store and release NPY at a high rate, to inhibit the melanotrope cells. In contrast, in black-adapted animals, the NPY-producing neurons in this nucleus are inactive, in keeping with the high rate of, uninhibited, α-MSH release from the melanotrope cells in

such animals (Jenks and Van Zoest, 1990).

Coexistence of neuropeptide Y with dopamine and GABA

Besides NPY, two other neuroregulatory factors inhibit release of α-MSH from the *Xenopus* melanotrope cell, namely DA and GABA (Verburg-Van Kemenade 1986a,b; 1987a). Since DA and GABA coexist with NPY within varicosities of the nerve plexus contacting the melanotropes (De Rijk et al., 1990a,1992; Van Strien et al., 1991), neurons that form all three of these factors are candidates for controlling the pars intermedia. The ventromedial thalamic nucleus and the ventral infundibular nucleus do not fulfill this criterion as they are negative with anti-DA and anti-TH (González et al., 1993; Tuinhof et al., 1993a). Similarly, the anterior preoptic nucleus and the posterior tubercle, which are DA-immunopositive (González et al., 1993; Tuinhof et al., 1993a), do not seem to innervate the pars intermedia as they are negative with anti-NPY (this study). In contrast, the suprachiasmatic nucleus appears to contain both DA and NPY, whereas also GABA-immunopositivity is present (R. Tuinhof, present study and unpublished observations). The present quantitative data on the effect of background adaptation on the occurrence and production of NPY, lead to the conclusion that in *X. laevis* the suprachiasmatic nucleus is involved in the inhibitory control of melanotrope cell activity in the pars intermedia.

This study is concerned with the role of hypothalamic neurons in the control of the pars intermedia. The possibility that, in addition to the hypothalamic suprachiasmatic nucleus, extrahypothalamic neurons control the pars intermedia, cannot be excluded. In this respect, the posterior thalamic nucleus might be a candidate as it is immunoreactive to anti-NPY (this study), anti-DA (González et al., 1993; Tuinhof et al., 1993a), and anti-GABA (R. Tuinhof, unpublished observations). However, the present data do not indicate such a role because in contrast to suprachiasmatic neurons, posterior thalamic neurons do not show a reaction to changed background light intensity, nor with respect to NPY-immunoreactivity (strong in both white- and black-adapted animals) or NPY-hybridization reaction (absent at both adaptation conditions).

In *Rana temporaria* DA and TH have been shown to be present in the preoptic region (González and Smeets, 1991), suggesting that in this amphibian the suprachiasmatic nucleus is also involved in the control of the pars intermedia In *Rana* direct connections exist between the retina and the preoptic area (Vullings and Heussen, 1975, Vullings and Kers, 1973) Therefore, it is tempting to assume that during background adaptation of amphibians information about background light intensity is directly transferred from the eye to the suprachiasmatic nucleus and from there, via NPY-producing neurons, to the melanotrope cells in the pars intermedia

ACKNOWLEDGEMENTS

The authors are greatly indebted to Dr D P V de Kleijn and Mr A J M Coenen for advice in the *in situ* hybridizations, to Dr G Pelletier and Dr H Vaudry for the gift of anti-neuropeptide Y and to Dr B G. Jenks for reading the manuscript Part of this research has been made possible by a grant from NWO/INSERM to E W R

CHAPTER 6

Coexistence of tyrosine hydroxylase and neuropeptide Y in suprachiasmatic neurons controlling background adaptation in *Xenopus laevis*: a confocal laser scanning microscopy study

Preliminary results from this study have been published as
Coexistence of neuronal messengers controlling melanotrope cells of *Xenopus laevis*
With Agustín González, Wilhelmus J.A.J. Smeets, Wim J.J.M. Scheenen and Eric W. Roubos in Eur. J. Morph. 32; 307-310 (1994)

With Ruud Ubink and Eric W. Roubos

ABSTRACT

The amphibian *Xenopus laevis* is able to adjust its skin color to the light intensity of its environment Paling of the skin is achieved by inhibiting the release of α-melanophore stimulating hormone (α-MSH) from the melanotrope cells of the pars intermedia of the hypophysis Neuropeptide Y, dopamine and GABA inhibit α-MSH-release These neurotransmitters occur in synapses on the melanotropes This study aims to support the hypothesis that the neurotransmitters are produced in neurons in the suprachiasmatic nucleus For this purpose immunofluorescence and confocal laser scanning microscopy were applied on free-floating sections of the *Xenopus* diencephalon It appears that the paired suprachiasmatic nucleus contains a medial and a lateral group of neurons that produce either neuropeptide Y or dopamine In the part of the caudolateral area of the suprachiasmatic nucleus that is situated most closely to the optic chiasm, 5-20 neurons were found that contain both neuropeptide Y and tyrosine hydroxylase It is concluded that these neurons are the regulators of the NPY-, dopamine- and GABA-mediated inhibition of melanotrope cell activity in *X laevis*

INTRODUCTION

The South African clawed toad, *Xenopus laevis,* can adjust the colour of its skin in response to stimuli, such as light, temperature and stress, in order to survive in its continuously changing environment (*e g* , Waring, 1963, Terlou et al , 1974) This adjustment depends on the amount of α-melanophore-stimulating hormone (α-MSH) produced by the melanotrope cells in the pars intermedia of the hypophysis The α-MSH causes darkening of the skin by stimulating dispersion of the black pigment melanin in skin melanophores Inhibition of α-MSH release leads to pigment aggregation and results in paling of the skin (*e g* Jenks et al , 1993) *In vitro* superfusion experiments with neurointermediate lobes have revealed that γ-aminobutyric acid (GABA), neuropeptide Y (NPY), dopamine (DA) and noradrenaline (NA) inhibit α-MSH release (Verburg-Van Kemenade et al , 1986a,b, 1987a) whereas corticotropin-

releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) stimulate this release (Verburg-Van Kemenade et al., 1987b,c).

CRH and TRH originate in the hypothalamic magnocellular nucleus (Tuinhof et al., 1994a) and have been shown immunocytochemically to be present in free axon terminals in the neural lobe of the hypophysis, from where they are thought to act upon melanotrope cells after their diffusion to the pars intermedia (Weatherhead, 1983; Jenks et al., 1993). NA has been demonstrated immunocytochemically in fibers in the pars intermedia as well as in perikarya in the locus coeruleus (González and Smeets, 1993). Neurons in the locus coeruleus and in the hypothalamic suprachiasmatic nucleus (SC) become labeled after application of retrograde tracers (DiI, DiA) into the pars intermedia (Tuinhof et al., 1994a). The inhibitory factors NPY, DA and GABA also occur in a fiber network in the pars intermedia. Furthermore, filling of the optic nerves with the tracer horseradish peroxidase has indicated the existence of a direct connection between the retina and NPY-producing suprachiasmatic neurons (Tuinhof et al., 1994a). At the ultrastructural level NPY, DA and GABA appear to coexist in synaptic contacts on the melanotropes (Van Strien et al., 1991; De Rijk et al., 1990a,1992). *In situ* hybridization showed the presence of *Xenopus* preproNPY-mRNA in suprachiasmatic neurons of animals adapted to a white background but not in black-adapted toads (Tuinhof et al., 1993b). Light microscopic immunocytochemistry using antisera raised to NPY (Lázár et al., 1993; Tuinhof et al., 1993b;1994c) and DA (González et al., 1993;1994) revealed the presence of two neuronal populations in the SC, one positive to anti-NPY and the other reacting to anti-DA.

All these findings together suggest that among the regulatory centers in the brain, the SC plays a particularly important role in the light-mediated inhibition of α-MSH-release during adaptation to a light background. The present study aims to substantiate this suggestion by studying if and which cell bodies in the SC show coexistence of NPY and DA. This has been done by immunocytochemistry involving anti-NPY and anti-tyrosine hydroxylase (TH) antisera and fluorescent antibodies conjugated with fluorescein isothyocyanate (FITC) and Texas Red. Then, the presence of the neurotransmitters was visualised in the same section using confocal laser scanning microscopy.

MATERIAL AND METHODS

Animals

Twelve adult (aged eight months) specimens of *Xenopus laevis*, weighing 28-32 g, were reared under standard laboratory conditions They were kept in tap water of 22°C under constant illumination and fed trout pellets (Trouvit, Trouw, Putten, The Netherlands) Animals were anaesthetised by immersion in a solution of 0 1% tricaine methane sulfonate (MS 222, Sandoz, Switzerland) and transcardially perfused with ice-cold 0 6% sodium chloride solution for 5 min, followed by a solution of ice-cold 4% paraformaldehyde in 0 1 M sodium phosphate buffer, pH 7 4 After removal from the skull, brains were postfixed in the same solution for 1-2 hrs and then immersed into 30% sucrose in 0 1 M sodium phosphate buffer (pH 7 4) at 4°C, for at least 16 hrs

Immunocytochemistry

Free-floating diencephalic sections were obtained by embedding cryoprotected brains (saturated in 30% sucrose) in a solution of 15% gelatine with 30% sucrose in 0 1 M sodium phosphate buffer, and stored in a 10% formaldehyde solution at 20°C for 5-7 hrs After rinsing in 30% sucrose, the gelatine blocks were cut on a cryostat at 60 µm thickness and transversal, horizontal and sagittal sections were collected in 0 1 M phosphate-buffered saline (PBS, pH 7 4) Rinses of 3 times 1 hr at 20 °C in 0 1 M PBS containing 0 3% Triton X-100 (Sigma, St Louis, MO, U S A) (PBSTX, pH 7 6) preceded the incubations Aspecific binding was prevented by rinsing the sections in PBSTX with 20% normal goat serum for 15 min at 20 °C The first incubation was performed in PBSTX for 48 hrs at 4°C in a cocktail of rabbit anti-NPY (1 1000, a kind gift from Dr H Vaudry, Rouen, France) and mouse anti-tyrosine hydroxylase (1 250, TH, Incstar, Almere, The Netherlands) The high specificities of the first antibodies, NPY and TH, have been reported previously (see Danger et al , 1985, González et al , 1993)

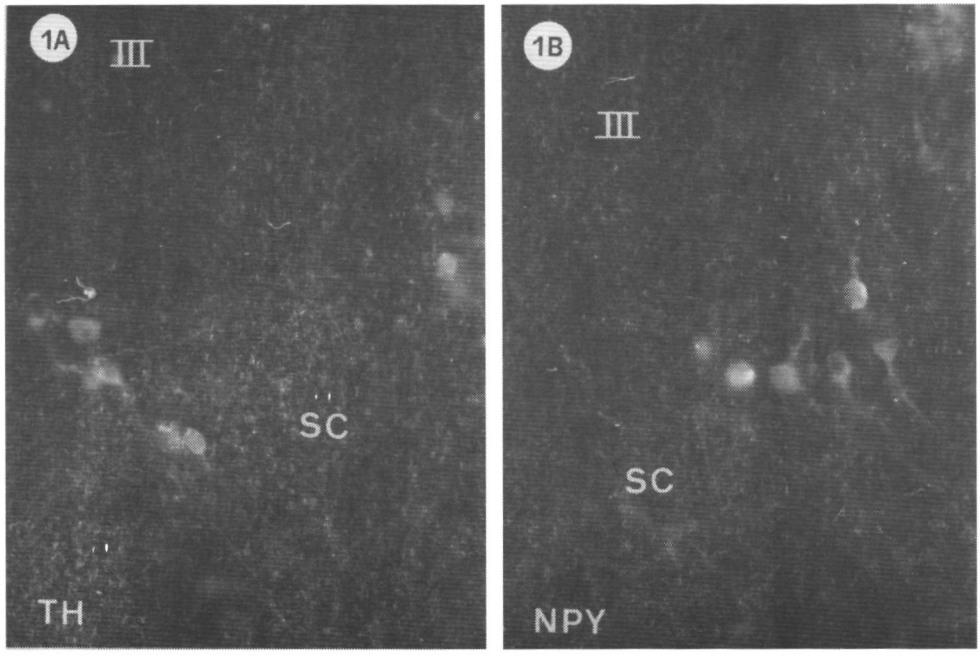


Fig. 1. Suprachiasmatic nucleus (SC) section in *Xenopus laevis*, showing cell bodies and fibers stained with mouse anti-TH (A) and rabbit anti-NPY (B). As second antisera, anti-mouse Ig, conjugated with Texas Red and anti rabbit Ig, conjugated with FITC were applied on the 30 μm thick sections to reveal the distribution of TH and NPY. The two populations in this medial part of the SC show no coexistence. *III*, third ventricle; *SC*, suprachiasmatic nucleus. Scalebar = 20 μm.

After rinsing, the sections were incubated in a cocktail of the second antibodies, conjugated with FITC (1:60; goat anti-mouse; Boehringer Mannheim; Mannheim, Germany) and Texas Red (1:100; donkey anti-rabbit; Amersham; Buckinghamshire, England), which was performed at 4°C for 48 hrs in the dark. Finally, the sections were rinsed in PBSTX and cover slipped in Citifluor (Agar Scientific Ltd, Stanstedt Essex, UK).

Confocal laser scanning microscopy

Sections were examined for double immunofluorescence staining with a confocal laser scanning microscope (Biorad MRC 600, Hemel Hampstead, UK) equipped with an argon-laser. A combination of the BHS, A2 (488 nm excitation

and 540 nm bypass filter) and the A1, A2 (514 nm excitation and 600 nm barrier filter) filter blocks was used to differentiate between separate FITC and Texas Red signals at photo multiplier tubes (PMT) The FITC (TH) was detected using PMT2, while the Texas Red (NPY) signal was detected through PMT1 Before collecting the Texas Red signal, the FITC signal was bleached by full power laser opening

RESULTS

With either single labeling or double labeling, immunopositive cells in the diencephalon were found in the SC The paired SC reveals a large number of neurons that is TH-immunoreactive but negative to anti-NPY These neurons are situated in a medial and a lateral group Also various NPY-positive but TH-negative neurons occur in these groups All of these neurons have a mean diameter of about 10-20 µm

In the medial group the NPY- and TH-positive neurons occur in small groups or are intermingled, but no indications for coexistence of NPY and TH within one neuron were obtained (Figs 1 and 2a,b) Many NPY-immunoreactive neurons are located close to the third ventricle, and some of them have dendrites that seem to be in contact with the cerebrospinal fluid

In the lateral group the populations of NPY-positive and TH-positive neurons overlap in such a way that 5 to 20 neurons reveal immunoreactivity to both antisera (Figs 2,3) These neurons are especially situated in the area of the SC that is closely apposed to the optic chiasm (Figs 2a,b) At higher magnifications different compartments can be discerned in these neurons and the proximal parts of their axons Some compartments show NPY-positivity and resemble the endoplasmic reticulum and/or Golgi apparatus (Fig 4), others exhibit anti-TH reactivity and occur scattered throughout the cytoplasm The neurons often show

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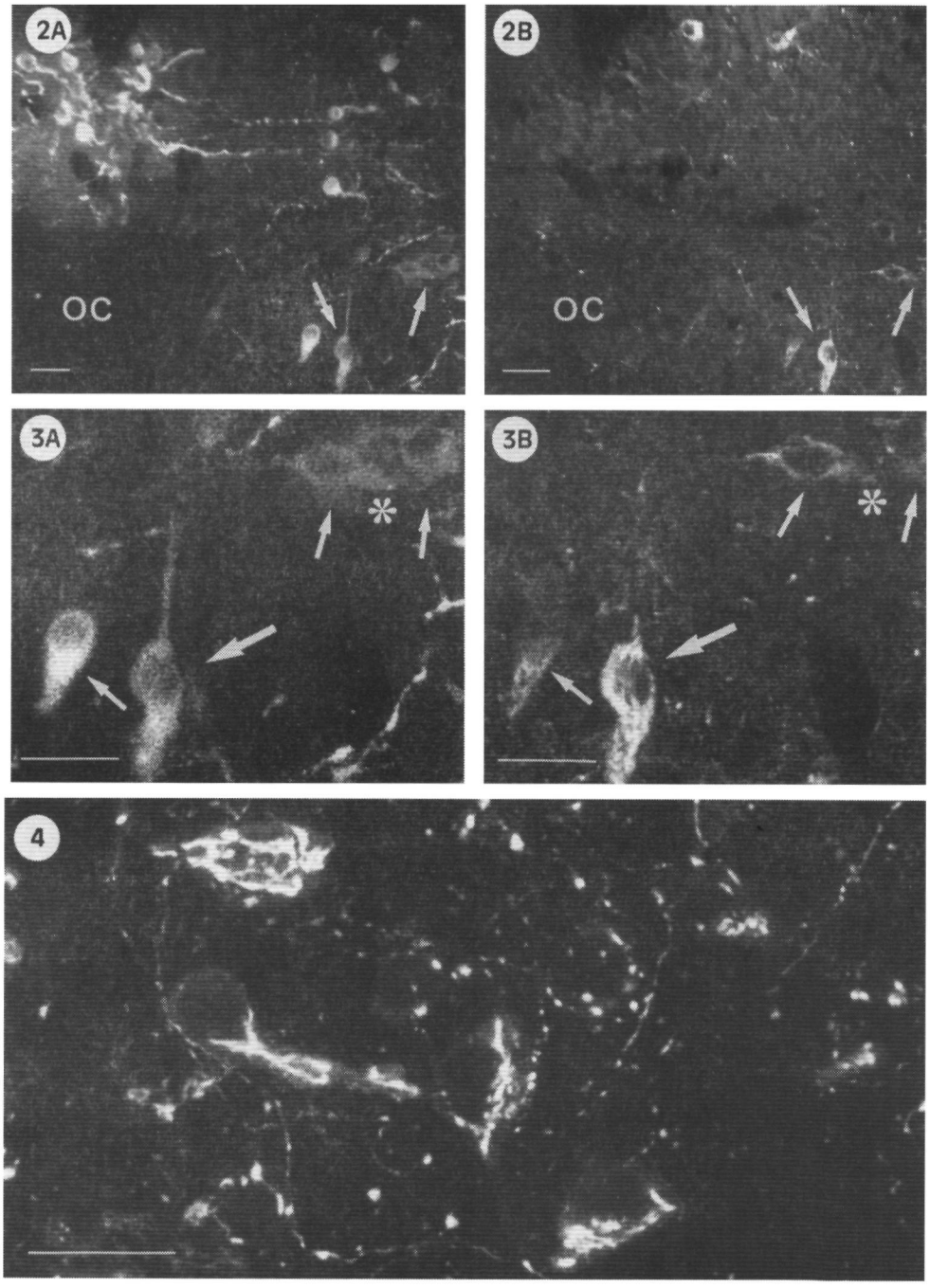
Figs 2-4. TH- and NPY-immunoreactive neurons and fibers in the suprachiasmatic nucleus **Figures 2** and **3** clearly show the coexistence of TH (**figs 2a,3a**, arrows) and NPY (**figs 2b,3b,** arrows) in neurons in the lateral part of the SC However, not in all cells NPY and TH coexist, *e g* the TH-positive cell (**fig. 2a***) is NPY-negative (**fig. 3a***) **Figure 4** shows the characteristic NPY distribution in suprachiasmatic neurons and axons *OC*, optic chiasm Scalebar = 20 µm


2A
OC
2B
OC
3A
*
3B
*
4

variable staining intensities with each of the antisera. In a section through the lateral group both moderately and strongly stained NPY-immunoreactive neurons can be observed (Figs 2b,3b) and in the same section, changing the excitation wavelength, a similar difference can be found with respect to anti-TH staining (Figs 2a,3a,b). Also, within the same perikaryon immunoreactivity to TH may strongly differ from that to NPY.

DISCUSSION

The activity of the melanotrope cells of *Xenopus laevis* is under the control of a direct synaptic innervation. At the ultrastructural level the synaptic contacts contain NPY, DA and GABA (Van Strien et al., 1991; De Rijk et al., 1990a,1992). This observation indicates that the perikaryal source of these synapses should be able to produce all three of the neural factors. According to this reasoning, the present study was carried out to support the hypothesis that the SC contains neurons that inhibit the melanotrope cells by releasing NPY, DA and GABA. Previously we have shown the presence of TH-immunoreactivity in suprachiasmatic neurons, indicating the ability of these neurons to produce DA (González et al., 1993, 1994; Tuinhof et al., 1993a) and we have also demonstrated the presence of NPY-immunoreactivity in suprachiasmatic neurons (Tuinhof et al., 1992, 1993b, 1994c). In these studies alternate sections had been stained with either anti-NPY or anti-TH serum. This approach did not permit us to draw conclusions about a possible coexistence of NPY and DA within one and the same SC neuron, for a number of reasons. For instance, the SC neurons are too small (about 15 μm in diameter) to allow for an appropriate number of sections to be cut. Furthermore, different sections may stained differently due to different interactions with the incubation media. In the present study we solved this problem by using dual wavelength immunofluorescence with different fluorochromes which provided the possibility to evaluate the presence of two antigens within the same section. To obtain maximal immunosensitivity, a free-floating section method was used, permitting optimal penetration of the antisera into the tissue sections. In the free-floating technique relatively thick (20-30 μm) sections have to be used, reducing the resolution power. However, this disadvantage was successfully overcome by applying confocal laser scanning microscopy. This technique permits the use of optical

sections with a thickness as thin as 1 μm even in fairly thick sections. Moreover, the digital images obtained (either induced by FITC- or by Texas Red-fluorescence) can be directly stored, thereby preventing the loss of fluorescence by bleaching The danger of bleeding of the two different signals was furthermore eliminated by bleaching out the FITC signal by full power laser opening before collecting the Texas Red signal (see also Brelje et al , 1993).

With the double immunofluorescence method using TH- and NPY-antisera, different populations of neurons have been localised, situated medially and laterally in the paired SC The cells that contain NPY and TH occur laterally, closely to the optic chiasm This observation indicates that these neurons are able to produce both NPY and DA, and that they are very likely the neurons that project to the pars intermedia to form synapses on the melanotrope cells. This supposition fits in with the observation that retrograde tracers injected into the pars intermedia of *Xenopus* label suprachiasmatic neurons that are located closely to the optic chiasm whereas cells in other parts of the SC do not become labeled (Tuinhof et al., 1994a). It is, moreover, in line with observations that GABA-immunoreactivity is not only present in the synapses in the pars intermedia but also in the amphibian SC, namely in *Rana esculenta* (Franzoni and Morino, 1989) and *X laevis* (A Fasolo, personal communication, R. Tuinhof, unpublished results) Therefore, we assume that the neurons found in the present study to contain NPY and DA, also contain GABA, and are responsible for inhibition of melanotrope cell activity by releasing these three transmitters Interestingly, on the basis of the observed staining differences with anti-NPY and anti-TH in their perikarya, it would seem that these particular SC neurons can produce and/or release NPY and DA with different strengths. This would seem to be in accordance with the observation that NPY and GABA (and DA) inhibit with different strengths α-MSH release from melanotrope cells *in vitro* (Leenders et al., 1993) This difference has been explained by assuming that the neurotransmitters may be differentially released depending on the state of adaptation of the animal Thus, for instance, GABA would act fast but only shortly, at the onset of adaptation to a light background, whereas NPY might take over during long-lasting adaptation conditions

The majority of the neurons staining with NPY did not show anti-TH immunoreactivity Still, there are strong indications that these neurons are also involved in the process of background adaptation to a light background For

instance, in animals adapted to a black background, no NPY-immunoreactivity was noticeable in the SC, neither was there any visible expression of prepro-NPY-mRNA after *in situ* hybridization with a *Xenopus*-specific probe In white-adapted animals, however, many neurons in the SC show NPY-immunoreactivity and mRNA expression (Tuinhof et al , 1993b) The question arises whether these neurons also project to the pars intermedia and form a separate projection that does not contain DA and GABA This does not seem likely, as nearly all synaptic contacts in the pars intermedia contain DA (and GABA) in addition to NPY (De Rijk et al , 1992) Nevertheless, it may be that the NPY-positive SC neurons that do not reveal TH-immunoreactivity, produce DA in the axon terminals rather than in their perikarya Also, the presently used free-floating method may be much more sensitive than the routine method used previously (Tuinhof et al , 1993b), revealing NPY-positive cells that previously appeared to be negative as their NPY contents were too low to be detected with the earlier method Alternately, it may be that these neurons do not project to the pars intermedia but have another role in the background-dependent adaptation process, such as being interneurons that mediate optic information to the SC neurons that contain NPY and TH and project to the melanotropes In the latter case, it might well be that particularly these TH-negative NPY-neurons are contacted by axons running from the optic tract (Tuinhof et al , 1994a)

ACKNOWLEDGEMENTS

The authors are greatly indebted to Miss P G P Wismans and Mr J Eygensteyn for technical assistance and Mr R J C Engels for animal care

CHAPTER 7

Involvement of retinohypothalamic input, the suprachiasmatic nucleus, the magnocellular nucleus and the locus coeruleus in neural control of melanotrope cells of *Xenopus laevis*: a retrograde and anterograde tracing study

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in Neuroscience ***61****; 411-420 (1994)*

ABSTRACT

The amphibian *Xenopus laevis* is able to adapt the colour of its skin to the light intensity of the background, by releasing melanophore-stimulating hormone from the pars intermedia of the hypophysis In this control various inhibitory (dopamine, γ-aminobutyric acid, neuropeptide Y, noradrenaline) and stimulatory (thyrotropin-releasing hormone and corticotropin-releasing hormone) neural factors are involved Dopamine, γ-aminobutyric acid and neuropeptide Y are present in suprachiasmatic neurons and coexist in synaptic contacts on the melanotrope cells in the pars intermedia, whereas noradrenaline occurs in the locus coeruleus and noradrenaline-containing fibers innervate the pars intermedia In the present study, the neuronal origins of these factors have been investigated using axonal tract tracing and immunocytochemistry Application of the lipophilic tracer DiI into the pars intermedia resulted in labeling neurons in both the suprachiasmatic nucleus and the locus coeruleus, indicating that both centers are involved in neural inhibition of the melanotrope cells Thyrotropin-releasing hormone and corticotropin-releasing hormone occur in axon terminals in the pars nervosa and were demonstrated immunocytochemically in the magnocellular nucleus These neurons were labeled following tracer application (DiI and horseradish peroxidase) into the pars nervosa This finding is in line with the idea that corticotropin-releasing hormone and thyrotropin-releasing hormone stimulate melanotrope cell activity after diffusion from the neural lobe to the pars intermedia After anterograde filling of the optic nerve with horseradish peroxidase, labeled axons were traced up to suprachiasmatic neurons that show positive staining with antisera raised to neuropeptide Y and tyrosine hydroxylase Possibly, a retino-suprachiasmatic tract is involved in the control of the melanotrope cells during the process of background adaptation

INTRODUCTION

The South African clawed toad, *Xenopus laevis,* is capable of adjusting the colour of its skin in response to environmental stimuli, such as light, temperature and handling stress (Jenks and Van Zoest, 1990, Roubos, 1992, Jenks et al , 1993) This adjustment is under control of α-melanophore

stimulating hormone (α-MSH) produced by the melanotrope cells in the pars intermedia of the hypophysis Stimulation of α-MSH release causes dispersion of melanin in the skin melanophores, resulting in blackening of the skin Inhibition of α-MSH release leads to aggregation of the pigment, giving the skin a pale appearance (Jenks and Van Zoest, 1990, Jenks et al , 1993) The release of α-MSH is regulated by various neural factors *In vitro* superfusion experiments with neurointermediate pituitary lobes have revealed that dopamine (DA), γ-aminobutyric acid (GABA), neuropeptide Y (NPY) and noradrenaline (NA) inhibit α-MSH release (Verburg-Van Kemenade et al , 1986a,b,c, 1987a), whereas corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) stimulate α-MSH-release (Verburg-Van Kemenade et al , 1987b,c) The origin of these factors is poorly known but there is circumstantial evidence that both hypothalamic and extrahypothalamic areas are involved Immunoelectron microscopy has shown that DA, GABA and NPY coexist in synaptic terminals that contact the melanotropes (De Rijk et al , 1992) and light microscopic immunocytochemistry using antisera raised to NPY (Lázár et al , 1993, Tuinhof et al , 1993b) and DA (González et al , 1993, Tuinhof et al , 1993a) suggests that the suprachiasmatic nucleus produces these inhibitors Moreover, *in situ* hybridization with a probe specific for *Xenopus* preproNPY-mRNA (Van Riel et al , 1993) revealed that suprachiasmatic neurons produce this mRNA in animals adapted to a white background but not in black background-adapted toads (Tuinhof et al , 1993b) NA has been demonstrated immunocytochemically in a nervous network in the pars intermedia as well as in perikarya in the brain stem, *i e* , in the locus coeruleus (González and Smeets, 1993) As to the stimulatory factors, CRH and TRH have been found immunocytochemically in axon terminals in the neural lobe of the hypophysis (Verburg-Van Kemenade et al , 1987b,c) It is believed that they stimulate melanotrope cell activity after diffusion from the neural lobe to the pars intermedia (Jenks et al , 1993, Verburg-Van Kemenade et al , 1987b,c) The origin of the CRH- and TRH-containing nerve fibers is not known

The first aim of the present study was to locate neuronal centers that control the activity of the melanotrope cells of *X laevis* The second aim was to get an impression of the pathway(s) by which optic information (as transmitted during background adaptation) is conveyed to such neuronal centers For these purposes retrograde tracers were introduced into the intermediate and neural

lobes of the hypophysis, whereas the optic nerves were anterogradely filled Neuronal centers were identified using immunocytochemistry

EXPERIMENTAL PROCEDURES

Animals

Young adult (aged eight months) specimens of *Xenopus laevis*, reared in our laboratory, with a weight of 28-32 g were used Full background adaptation was achieved by keeping the animals for three weeks on a white background, at 20 °C and constant illumination Animals were fed trout pellets (Trouvit, Trouw, Putten, The Netherlands) Prior to the experiments all toads were anaesthetized with 0 1% tricaine methane sulfonate (MS 222, Sandoz, Switzerland)

Retrograde tracing with DiI

Sixteen animals were transcardially perfused with ice-cold 0 6% Ringer solution for 5 min followed by perfusion fixation with 4% paraformaldehyde (PFA) for 15 min Brains of 8 animals were dissected out and fixed in the same PFA fixative for 2 h For tracing, a tiny crystal of DiI (1,1' dioctadecyl-3,3,3',3' tetramethylindocarbocyanine, Molecular Probes, Eugene, OR, U S A) was introduced unilaterally, halfway into the pars intermedia or the pars nervosa (see refs 11,51) Then the brains were stored in the dark in 4% PFA, for 2-4 weeks, at 36 °C, and subsequently cryoprotected in 30% glucose in sodium phosphate buffer, for 24 h Tissues were frozen and cut horizontally, sagittally or transversally into 10-20 µm sections with a freezing microtome Sections were transferred to poly-L-lysine-coated glass-slides and air-dried

The brains of the remaining 8 animals were immersed *in situ* for 16 h in the PFA fixative Then DiI was applied as described above Two weeks later the brains were dissected and cut on a vibratome in 50 µm coronal sections that were mounted in glycerol They were examined with a Leitz microscope equipped with a Leitz I2 filter

Retrograde tracing with HRP

In five anaesthetized animals a small opening was made in the roof of the

mouth, and a small crystal of horseradish peroxidase (HRP; Sigma, St Louis, MO, U.S A.) was introduced unilaterally, halfway into the pars intermedia or the pars nervosa. After five days the animals were re-anaesthetized and their brains fixed. For this purpose perfusion was carried out with, consecutively, 0 6% Ringer solution, for 5 min, and a mixture of 4% PFA, 0.05% glutaraldehyde and 0 2% picric acid in 0.1 M sodium phosphate buffer, for 15 min. Then the brains were dissected out, fixed in the same mixture for 2 h, cryoprotected in 30% glucose in phosphate buffer for 24 h, and cut, mounted and dried as described above.

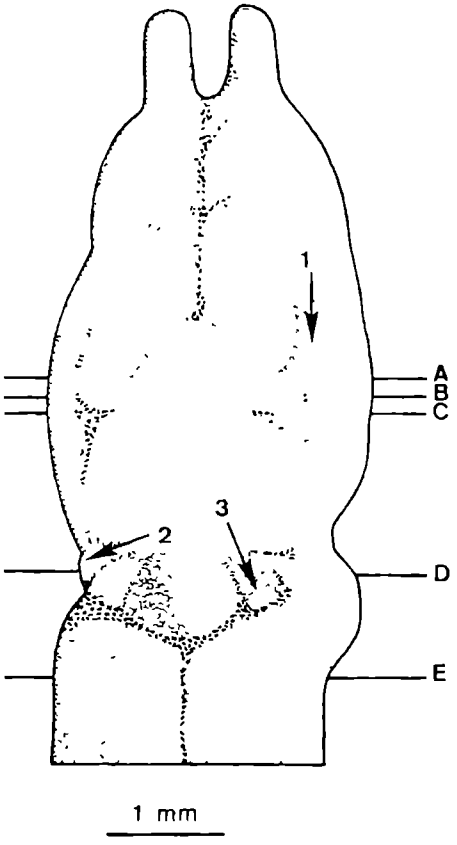


Fig. 1 Ventral view of the brain of the South African clawed toad *Xenopus laevis*, in capitals representing the levels at which transversal sections have been presented in **figure 2.** Numbers point at the injection sites of anterograde and retrograde tracers, 1 HRP in the optic nerve, 2 HRP and DiI into the pars nervosa and 3, HRP and DiI into the pars intermedia

Anterograde tracing with HRP

In 8 anaesthetized animals the roof of the mouth was opened and the left optic nerve was cut. Its central stump was exposed to a small crystal of HRP. Then the animals and brains were treated as described for retrograde HRP tracing.

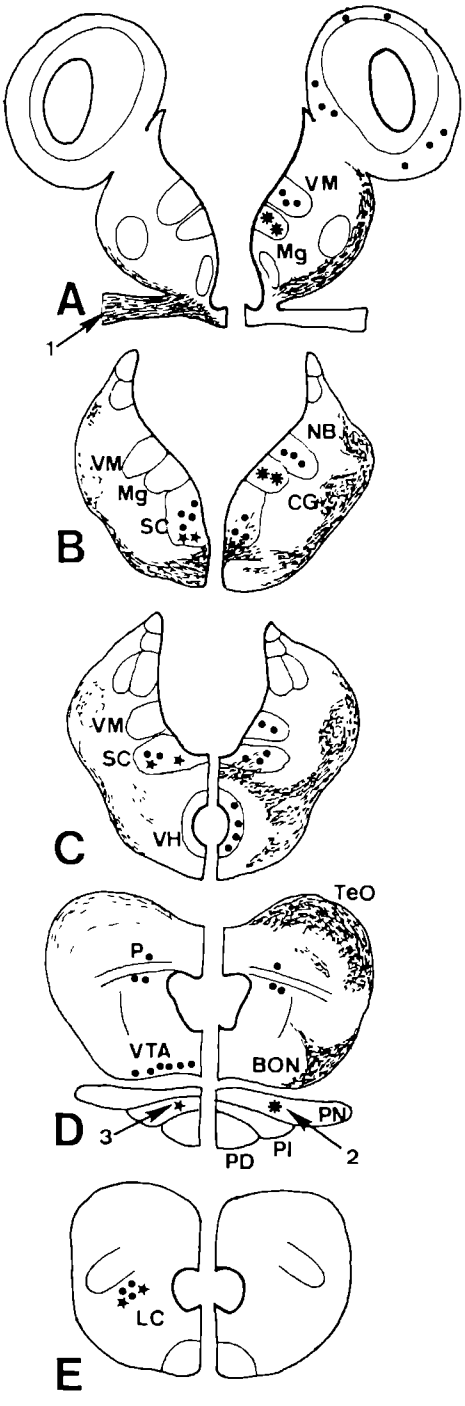


Fig. 2. Diagram of transversal sections through the brain of *Xenopus laevis*, at levels indicated in **figure 1**. Dots on the left represent TH-immunoreactive neurons and on the right NPY-positive neurons The wavy lines represent filled fibers after application of HRP into the optic nerve (1). Retrograde labeling after injection of DiI in the PN (2) and PI (3) is also indicated with different stars. *BON*, basal optic nucleus; *CG*, geniculate body; *LC*, locus coeruleus; *Mg*, magnocellular nucleus; *NB*, nucleus of Bellonci; *P*, posterior thalamic nucleus; *PD*, pars distalis; *PI*, pars intermedia; *PN*, pars nervosa; *SC*, suprachiasmatic nucleus; *TeO*, optic tectum; *VH*, ventral infundibular nucleus; *VM*, ventromedial nucleus; *VTA*, ventral tegmental area.

Staining

To reveal HRP-filled optic projections, sections were rinsed in Tris-buffered saline (Tuinhof et al., 1993b) and Tris-HCl, and stained with nickel-enhanced DAB giving the filled optic fibers a bluish-black appearance. Then sections were stained with anti-NPY (rabbit; 1:4000), anti-CRH (rabbit; 1:300), anti-TRH (rabbit; 1:400) or anti-tyrosine hydroxylase (TH, mouse; 1:1000; Incstar; Amsterdam; The Netherlands), for 60 h incubation at 4 °C. As a secondary antiserum a goat anti-rabbit (1:100; Sigma) or goat anti-mouse (1:100; Sigma) serum was used, for 2 h at 20 °C. Finally, a rabbit or mouse peroxidase-antiperoxidase was applied (1:1000; Nordic, Tilburg, The Netherlands) followed by staining with 0.04% DAB (Sigma) and 0.015% H_2O_2 in 50 mM Tris-HCl (pH 7.6), for 40 min, giving immunopositive cells a brownish colour. Sections were studied with bright field microscopy.

Air-dried sections of brains were examined as described above. In some cases alternate sections of DiI-filled material were stained immunocytochemically.

RESULTS

The results obtained with both tracers are presented in transverse projection figures of the respective brain areas (Figs 1, 2). The nomenclature follows that previously used (González et al., 1993; Tuinhof et al., 1993b).

Retrograde tracing from the pars intermedia

Application of a DiI or HRP crystal to the pars intermedia resulted in all brains in a strongly stained fibre tract that could be traced back to the hypothalamus. The tract was paired, as appears from fillings of either the left or the right part of the pars intermedia. The fibers were visible throughout the pars intermedia, the only site where they show varicosities (Fig. 3), in the neural lobe (close to the pars intermedia), in the median eminence and in a tract running through the bottom of the hypothalamus underneath the ventral infundibular nucleus (VH). Just before the optic chiasm the tract bent dorsally between the chiasmatic ridge and the VH (Fig. 4), partly crossed the rostral tip of the VH. Dorso-rostrally directed fibers left the tract and ran to the suprachiasmatic

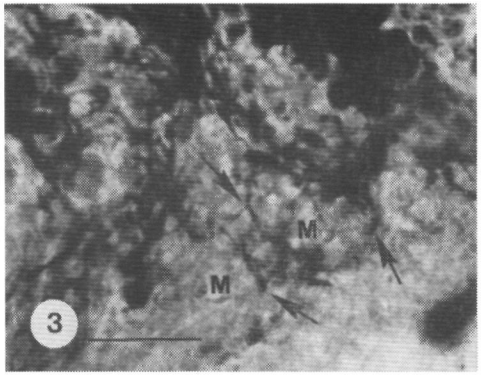
nucleus (SC). In this nucleus, about 10 to 20 neuronal perikarya appeared to be labeled (Figs 4,5). All labeled cells were ipsilaterally located with respect to the injection site. The neurons had a similar size of about 10-20 μm in diameter. The SC contained neurons that stain with both anti-TH (Fig. 6) and anti-NPY. The other fibers in the tract ran over the VH into a caudodorsal direction to the brainstem. Here some scattered, fluorescent (or HRP stained) perikarya (diameter about 10-20 μm) were found in an area corresponding to topographically the mammalian locus coeruleus (González and Smeets, 1993; fig. 7). Accordingly they will be called locus coeruleus (LC) neurons. The LC is a paired nucleus containing fluorescent cells, which had a diameter of 10-20 μm. Perikarya in the LC appeared to stain with anti-TH (Fig. 8).

Finally, in a minor number of cases, after injection of DiI into the pars intermedia a few neurons in the magnocellular nucleus appeared to be stained.

Retrograde tracing from the pars nervosa

Examination of brains of which the pars nervosa had been treated with tracer, either DiI or HRP, showed a strongly stained fibre tract that could be traced from the injection site to a rostral and finally dorsal direction, up to the magnocellular nucleus, where about 5-10 fluorescent perikarya were present (Fig. 9). They had a mean diameter of about 30-40 μm and showed a positive reaction with anti-CRH and anti-TRH (Fig. 10).

Fig. 3. HRP injection site in the pars intermedia Note the nickel-enhanced DAB staining of the anterogradely filled varicose fibers (arrows) that surround the melanotrope cells (M, not specifically stained) Scalebar = 25 μm **Fig. 4** Fluorescent perikarya in the suprachiasmatic nucleus (SC) after retrograde DiI-filling from the pars intermedia (PI) in a sagittal section through the hypothalamus The DiI-labeled tract (star) runs dorsally between the ventral infundibular nucleus (VH) and the optic chiasm (OC) Scalebar = 50 μm **Fig. 5** Transversal diencephalic section showing DiI-labeled suprachiasmatic neurons and fibers after injection of DiI in the pars intermedia Scalebar = 50 μm **Fig. 6** TH-immunoreactive neurons and fibers in a transverse section through the suprachiasmatic nucleus (SC) Scalebar = 50 μm **Fig. 7** Transversal section of DiI-positive neurons and fibers in the locus coeruleus (retrograde filling from pars intermedia) Scalebar = 50 μm **Fig. 8** TH-immunoreactive perikarya and fibers in a transversal section through the locus coeruleus Scalebar = 50 μm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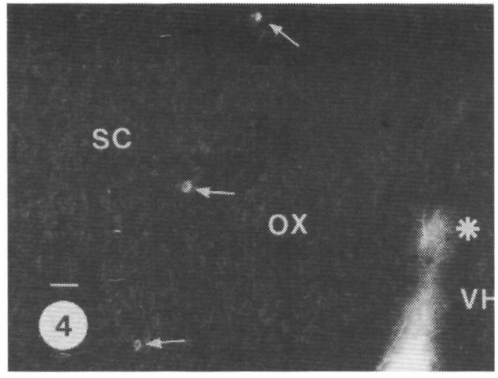
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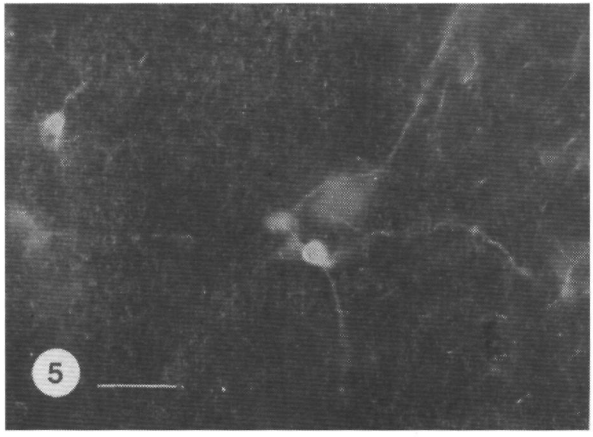
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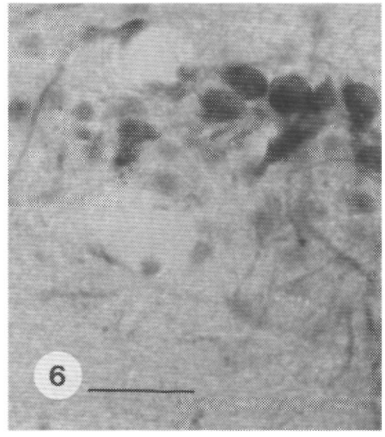
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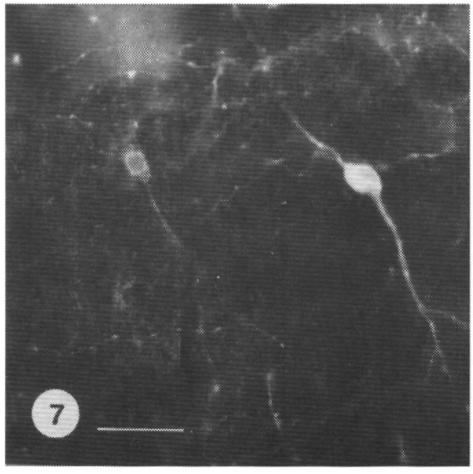
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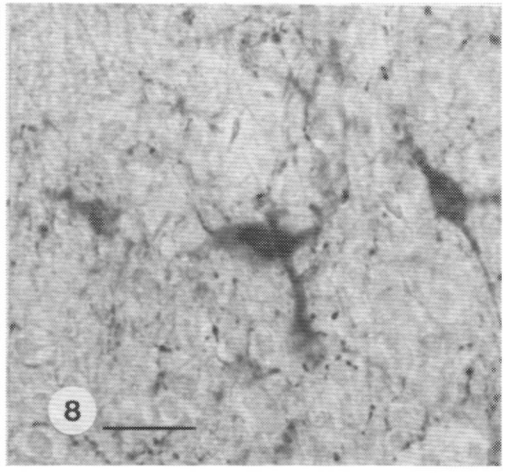
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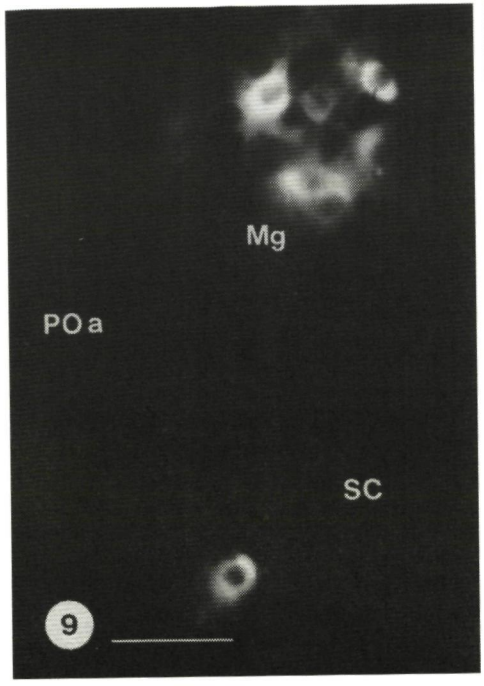

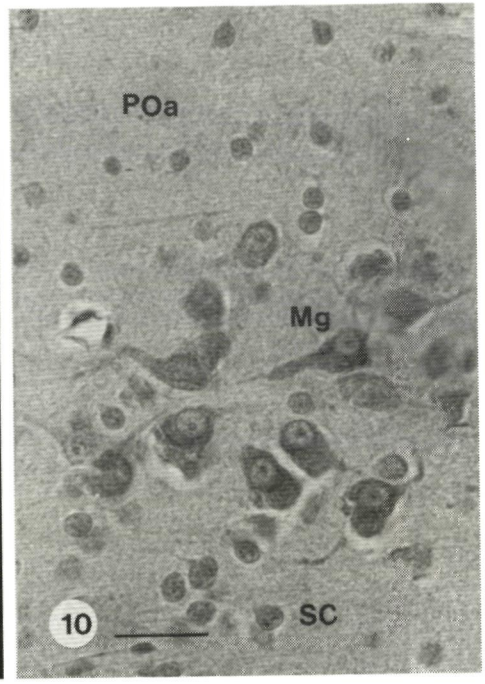

Fig. 9. Neurons in the magnocellular nucleus (Mg) in a sagittal section show a positive fluorescence after application of DiI in the PN. *POa,* anterior preoptic nucleus; *SC,* suprachiasmatic nucleus. Scalebar = 100 µm. **Fig. 10**. TRH-immunoreactive neurons in a horizontal section through the magnocellular nucleus (Mg). *POa,* anterior preoptic nucleus; *SC,* suprachiasmatic nucleus. Scalebar = 100 µm.

Tracing of the optic nerve

Upon labeling the optic nerve, a small ipsilateral projection was seen in the optic tectum (TeO), the geniculate body (CG) and the nucleus of Bellonci (NB). However the majority of HRP-filled fibers projected via the optic chiasm to the contralateral side of the diencephalon and mesencephalon. The main contralateral projection area was the TeO (Fig. 11). Other contralateral projections from rostral to caudal included the NB, the CG (Fig. 12), the SC, the pretectal area and the basal optic nucleus.

In particular, some fibers of the tract that end in the SC showed various varicosities, many of which could be seen to be in close contact with suprachiasmatic perikarya (Fig. 13). These perikarya had a mean diameter of 10-

20 µm and stain with anti-NPY (only in white-adapted animals; Fig. 13) and anti-TH.

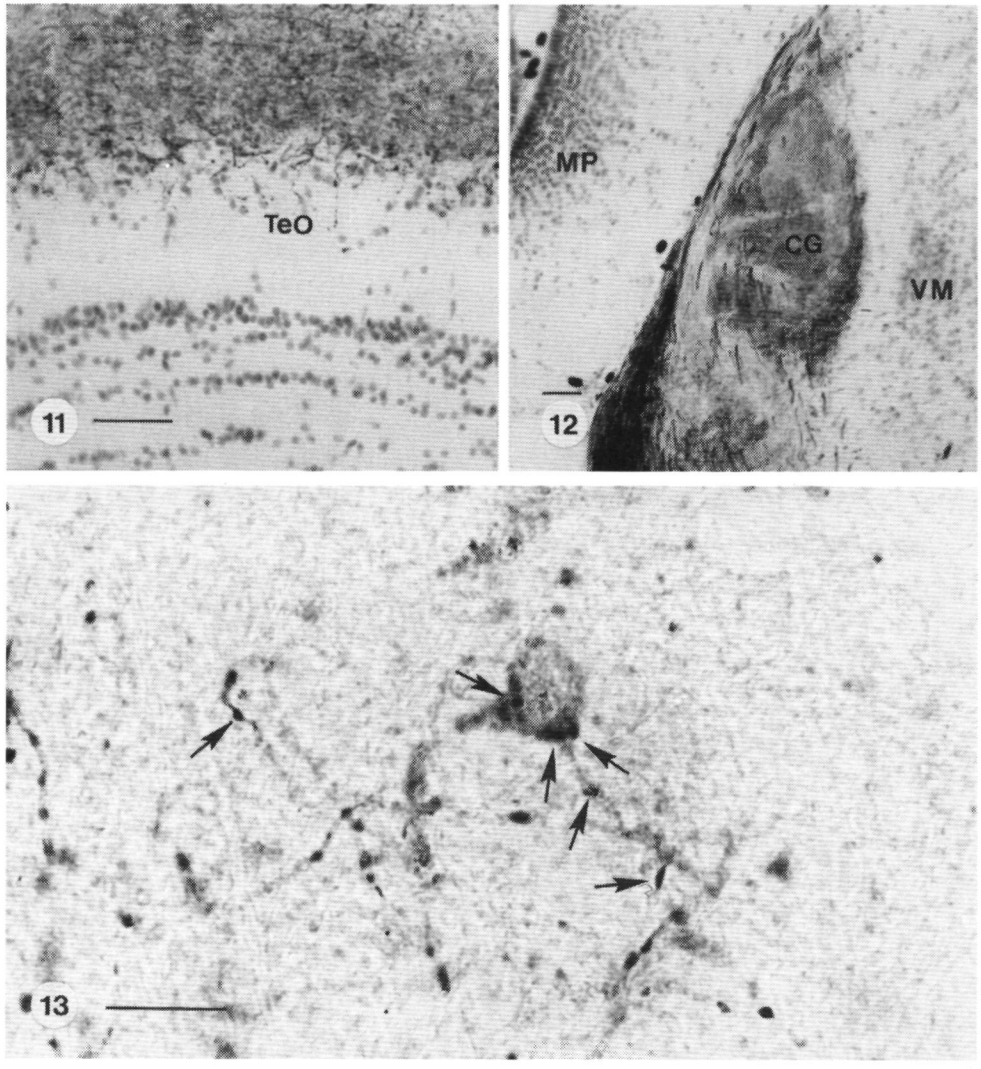

Fig. 11. HRP-positive innervation of the contralateral optic tectum (TeO). Scalebar = 100 µm. **Fig. 12**. HRP-containing fibers after labeling the optic nerve. In a transversal section through the diencephalon a strong contralateral innervation is seen in the geniculate body (CG). *MP*, medial pallium; *VM*, ventromedial nucleus. Scalebar = 100 µm. **Fig. 13**. Horizontal section through the suprachiasmatic nucleus. HRP filling of the optic nerve. Arrows indicate dark (HRP-positive) axons with varicosities abutting an NPY-positive perikaryon and its axon processes. Scalebar = 50 µm.

DISCUSSION

Suprachiasmatic innervation of the pars intermedia

Immunocytochemistry at the light and electron microscope level has shown that the pars intermedia of the hypophysis of the aquatic toad *Xenopus laevis* is innervated by an intricate network of nerve fibers that make synaptic contacts with the melanotrope cells (Verburg-Van Kemenade 1987a, De Rijk et al , 1990a,b) In the present approach individual fibers of this network have been retrogradely filled with DiI up to their perikarya in the SC Therefore we conclude that suprachiasmatic neurons are involved in synaptic control of the melanotrope cells This conclusion is in line with previous studies that have shown that suprachiasmatic neurons in *Xenopus* express preproNPY-mRNA and are NPY-immunoreactive in animals adapted to a white background but not in animals adapted to a black background (Tuinhof et al , 1993b) Apparently, NPY produced in the SC inhibits α-MSH release from the melanotropes during adaptation to a white background This results in paling of the skin

In many preparations a restricted number of DiI-labeled fluorescent fibers were seen to pass the neural lobe before entering the pars intermedia This is not surprising as some NPY-positive fibers have been shown in this area as well (De Rijk et al , 1990a) They can be considered to run 'en passant' through the neural lobe on their way to the pars intermedia

There is reason to believe that the role of the SC in the control of the pars intermedia is not restricted to *Xenopus*, because like the situation in *Xenopus* (De Rijk et al , 1992), colocalization of inhibitory neurotransmitters in the terminal network of the pars intermedia has also been demonstrated in *Rana* (Tonon et al , 1992) Furthermore both in *Xenopus* and *Rana* NPY (Andersen et al , 1993, Danger et al , 1985, Lázár et al , 1993, Tuinhof et al , 1993b), DA (Gonzalez and Smeets, 1991, Carr et al , 1992, Tuinhof et al , 1993a) and GABA (Franzoni and Morino, 1989) coexist in the SC Moreover, after retrograde labeling of the pars intermedia with DiI, labeled neurons have been shown in the suprachiasmatic hypothalamus of *R esculenta* and the urodele *Triturus carnifex* (Artero et al , 1994)

A retino-suprachiasmatic pathway

The present anterograde labeling of fibers in the optic nerve has revealed labeled axons and varicosities close to suprachiasmatic neurons. Most likely, these suprachiasmatic neurons are projecting to the pars intermedia, as they stain with anti-NPY and with anti-TH, indicating the co-storage of NPY and DA, as has been found in the synaptic contacts in the pars intermedia (De Rijk et al., 1992). Therefore, it is very likely that the control of the suprachiasmatic neurons during background adaptation is, at least partly, effectuated by a direct retino-suprachiasmatic pathway.

Retinosuprachiasmatic pathways have previously been proposed to exist in *X. laevis* (Levine, 1980; Tóth et al., 1980) as well as in other amphibians, including *Bufo marinus* (Wye-Dvorak et al., 1992), *Rana esculenta* (Lázár and Székely, 1969; Lázár, 1978), *Rana pipiens* (Hughes and Hall, 1986; Montgomery and Fite, 1989) and *Rana temporaria* (Vullings and Heussen, 1975; Vullings and Kers, 1973). However, in all these cases the postsynaptic perikarya have not been characterized, nor have their neurotransmitter content, their axonal projections or their target cells.

Locus coeruleus innervation of the pars intermedia

In brains in which the pars intermedia was filled with DiI, fluorescent perikarya were observed in an area defined as locus coeruleus (González and Smeets, 1993), suggesting that this brain stem region is involved in the neural control of the melanotrope cells. This conclusion is in line with the fact that both perikarya in this nucleus and nerve fibers in the pars intermedia stain with anti-NA (González and Smeets, 1993). *In vitro*, NA inhibits the release of α-MSH from neurointermediate lobes kept in superfusion (Verburg-Van Kemenade, 1986c). Therefore we propose that, in addition to the SC, the locus coeruleus is involved in the inhibitory control of the melanotrope cells of *X. laevis* Possibly, this situation holds for amphibians in general because DiI application to the pars intermedia of the anure *R. esculenta* and the urodele *Triturus carnifex* also results into labeling of neurons in the brain stem (Artero et al., 1994).

As no labeled optic afferents were found in the locus coeruleus region, it remains to be seen whether this area plays a role in light-controlled background

adaptation. Another possibility is that the locus coeruleus neurons projecting to the pars intermedia play a role in stress-dependent regulation of the secretion of α-MSH. Involvement in the central mediation of stress stimuli for the locus coeruleus has been proposed for the rat (Tilders and Berkenbosch, 1986).

Magnocellular nucleus and background adaptation

CRH and TRH are believed to exert their stimulatory effect on α-MSH-release by diffusing from the neural lobe to the pars intermedia (Verburg-Van Kemenade, 1987b,c; Jenks et al., 1993).

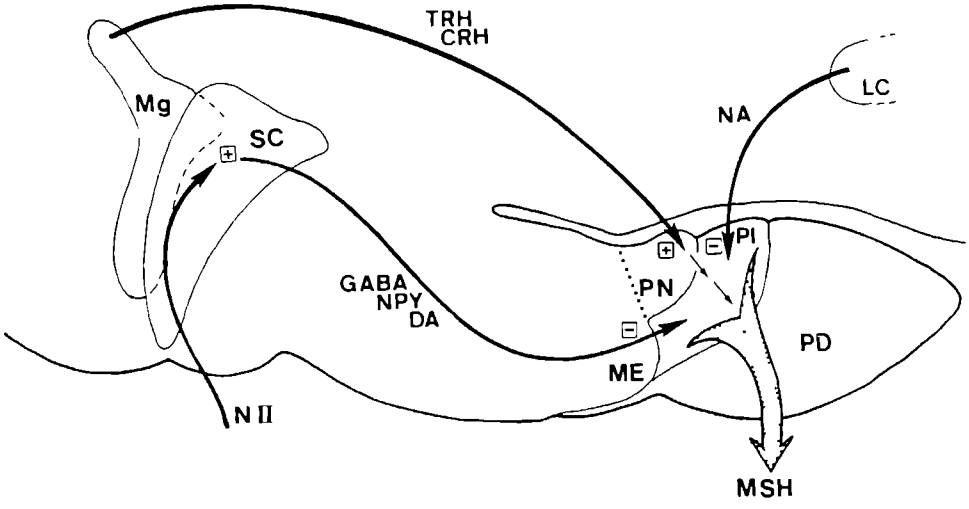

Fig. 14 Schematic representation of the regulation of the α-MSH release, projected in a midsagittal section of the central nervous system of *Xenopus laevis* *CRH*, corticotropin releasing hormone, *DA*, dopamine, *GABA*, γ-aminobutyric acid, *LC*, locus coeruleus, *ME*, median eminence, *Mg*, magnocellular nucleus, *α-MSH*, melanophore stimulating hormone, *NA*, noradrenaline, *NII*, optic nerve, *NPY*, neuropeptide Y, *PD*, pars distalis of the hypophysis, *PI*, pars intermedia of the hypophysis, *PN*, pars nervosa of the hypophysis, *SC*, suprachiasmatic nucleus, *TRH*, thyrotropin releasing hormone

In the present study we demonstrate that both peptides occur in hypothalamic magnocellular neurons TRH-mRNA, moreover, has been shown in all preoptic nuclei of *Xenopus*, including in the magnocellular nucleus (Zoeller and Conway, 1989) In this study we show that magnocellular neurons become labeled upon tracer application (DiI and horseradish peroxidase) to the neural lobe This finding supports the idea that the CRH- and TRH-producing centers that stimulate melanotrope secretory activity are located in the magnocellular nucleus This situation does not seem to hold for amphibians in general, as shown by immunocytochemical data on *R esculenta* Thus, whereas in *Rana* CRH and TRH are present in the magnocellular nucleus (Mimnagh et al , 1987, Seki et al , 1983, Tonon et al , 1980, 1985, Verhaert et al , 1984) and this nucleus innervates the neural lobe (Pasquier et al , 1980), TRH- and CRH-positive fibers penetrate the pars intermedia and therefore seem to control the melanotrope cells in a synaptic way (Andersen et al , 1993, Lamacz et al , 1989) Injection of DiI into the pars intermedia occasionally resulted in staining of (a few) neurons in the magnocellular nucleus This suggests that the magnocellular neurons are involved in the synaptic control of the pars intermedia It is unlikely that this control would involve CRH or TRH as no CRH- or TRH-immunoreactivity has been demonstrated in the pars intermedia On the other hand, in amphibians the magnocellular nucleus is a rich source of vasotocin (Vandesande and Dierickx, 1976, Carr and Norris, 1990, González and Smeets 1992a,b) Since vasotocin-immunoreactivity has been described in the pars intermedia of *Rana* (Vandesande and Dierickx, 1976), vasotocin-producing neurons might be involved in synaptic control of melanotropes in amphibians As to *Xenopus*, vasotocinergic innervation of the pars intermedia is very scanty or absent (Conway and Gainer, 1987, González and Smeets 1992b, C Artero, own observations) Therefore, in *Xenopus*, vasotocinergic magnocellular neurons do not seem to play an important role in the control of melanotrope cell activity This is substantiated by the fact that *in vitro* superfusion experiments do not show any effect of vasotocin on α-MSH release (Jenks and Verburg-Van Kemenade, 1988)

Other nuclei involved in the control of the pars intermedia of amphibians?

Over the years a number of other brain centers has been suggested also to

innervate the pars intermedia of tadpoles as well as adult frogs, namely the paraventricular organ in *R. temporaria* (Prasado Rao, 1982) and *X. laevis* (Terlou and Ploemacher, 1973; Terlou and Van Kooten, 1974), the preoptic recess organ (PRO) in *Bufo japonicus* (Kato et al., 1992) and *R. temporaria* (Prasado Rao, 1982) and the infundibular area in *R. catesbeiana* (Carr et al., 1991) and *R. temporaria* (Prasado Rao, 1982). As to *Xenopus*, these centers do not seem to play an important (quantitative) role in this regulation, as we did not observe their retrograde labeling after tracer application to the pars intermedia. Moreover, in *Xenopus* the PRO and PVO are a less likely sites of origin of axons innervating the pars intermedia because they lack NPY-positive neurons (Lázár et al., 1993; Tuinhof et al., 1993b) and the ventral infundibular nucleus is TH- and DA-negative (González et al., 1993).

Conclusion

Our data indicate that regulation of the melanotrope cells in the pars intermedia of *X. laevis* is exerted by at least three nuclei in the central nervous system. CRH and TRH produced in the magnocellular nucleus may stimulate whereas NPY, DA and GABA originating from the SC and NA produced in the locus coeruleus may inhibit the melanotropes. Since the suprachiasmatic NPY production depends on the light intensity of the background and the suprachiasmatic neurons appear to receive a direct input from the optic nerve, it is proposed that the suprachiasmatic nucleus is involved in the transduction of photic stimuli into signals that inhibit melanotrope cell activity (Fig. 14). The physiological stimuli controlling the magnocellular and locus coeruleus neurons remain to be investigated.

ACKNOWLEDGEMENTS

The authors are greatly indebted, for the gift of antibodies to Dr H. Vaudry and Dr G. Pelletier (anti-NPY), Dr E.A. Linton (anti-CRH) and Dr Th.J. Visser (anti-TRH), for technical assistance to Mrs R. de Boer-Van Huizen and Mr A.J.M. Coenen, and to Dr B.G. Jenks for critically reading the manuscript.

CHAPTER 8

General discussion

Background adaptation as a model for studying neuroendocrine regulatory processes

The objective of the work presented here is to increase insight into the fundamentals of neuroendocrine communication between the brain and the hypophysis, with emphasis on the regulatory processes that are responsible for the translation of environmental stimuli into adequate adaptive physiological responses. In our department, the physiological process of adaptation of the amphibian *Xenopus laevis* to the light intensity of its environmental background is being used as an experimental object to study the basics of neuroendocrine communication. In this context, this thesis concerns the localization in the *Xenopus* brain of the centers that regulate the activity of the melanotrope cells in the pituitary pars intermedia. These cells are the ultimate controllers of skin colour. In the following discussion some attention is first paid to the previous history and to the multidisciplinary approach that has been followed during the thesis research. Then the results are discussed and conclusions drawn. Finally, some issues originating from this work are put forward.

History and multidisciplinary approach

During the last three decades, the way of innervation of the amphibian melanotrope cells in the intermediate hypophysis has received considerable attention. Nevertheless, at the start of this research the nature of the regulatory centers was still a matter of dispute (see Pehleman, 1967; Oshima and Gorbman, 1969; Terlou et al., 1974; Carr et al., 1991). A rough indication had been obtained by lesion studies of the preoptic nucleus. As such lesions had no effect on the availability of the animal to adapt to a light background by paling its skin, it was concluded that the putative inhibitory hypothalamic centre would be located behind the optic chiasm (see Dierikcx, 1967). Subsequently, on the basis of various techniques particularly aiming at the identification of the putative inhibitory regulation of melanotrope cell activity (α-MSH release), a number of hypothalamic nuclei was proposed to be involved (e.g. the paraventricular organ; Terlou and Ploemacher, 1973). However, no conclusive evidence was presented.

During the last decades various techniques have become available to neuroscientists to elucidate neural connectivities and the functions of brain centers in physiological processes Several of these techniques have been used in this research, in order to assess the neuroendocrine involvement of background adaptation in *Xenopus* The main techniques are listed below

1) The availability of specific antibodies against neural factors proposed in melanotrope cell control enabled a detailed immunocytochemical investigation of the distribution of these neurotransmitters in the adult and developing *Xenopus* brain
2) The functioning of hypothalamic cell bodies in animals kept under different background light conditions was studied quantitatively by automated video-image analysis, using immunocytochemistry with an antiserum against NPY and *in situ* hybridization using a preproNPY-mRNA-probe
3) Retrograde and anterograde tracers offered the possibility to demonstrate connectivities between the optic nerve, the hypothalamus and the hypophysis
4) With recently developed fluorochromes colocalization studies were carried out of multiple neurotransmitters within neurons in the same tissue section Confocal laser scanning microscopy provided the opportunity to use relatively thick (60-100 µm) sections, which were stained by the free-floating method and finally were analysed by scanning them in 1 µm thick optical planes
5) The availability of an accurate timetable for the development of *Xenopus laevis* (Nieuwkoop and Faber, 1967) enabled to study the relationship between the ontogeny of NPY- and DA-immunoreactive systems and the development of the capacity of *Xenopus* to adapt to the light intensity of its background

On the basis of *in vitro* superfusion studies with neurointermediate lobes, various neural factors have been identified that are able to affect the rate of release of α-MSH from *Xenopus* melanotrope cells (Verburg-Van Kemenade 1986a,b, 1987a) Subsequently, these factors have been shown to be present in a terminating network in the pars intermedia of the hypophysis (Van Strien et al , 1991, De Rijk et al , 1992), supporting the idea that they are actually controlling

the activity of the melanotropes *in vivo*. In the present study these factors have been localized in a number of brain centers and in the case of inhibitory factors, their relation with changes in environmental light conditions and optic input on the one hand and with the control of the melanotrope cells on the other hand has been established.

Magnocellular nucleus

The melanotrope cells have been reported to increase the release of α-MSH when superfused neurointermediate lobes are treated with CRH (Verburg-Van Kemenade, 1987c) or with TRH (Tonon et al., 1980; Leroux et al., 1982; Verburg-Van Kemenade, 1987b). TRH has been found in the magnocellular nucleus (Mg) of *Rana catesbeiana* (Seki et al., 1983; Mimnagh et al., 1987) and of *Xenopus laevis* tadpoles (Goos 1978), and in the same nucleus CRH-containing neurons are found, namely in *Rana ridibunda* (Verhaert et al., 1984; Tonon et al., 1985), *Rana catesbeiana* (Lederis, 1987; Gonzalez and Lederis, 1988), *Triturus cristatus carnifex* (Fasolo et al., 1984) and *Xenopus laevis* (Olivereau, 1987). In the present study the presence of TRH and CRH in the Mg of *Xenopus* has been confirmed (Chapter 7). The CRH- and TRH-positive hypothalamo-hypophyseal tracts terminate predominantly in the median eminence and in the pars nervosa. TRH innervation of the pars intermedia has been reported in *R. catesbeiana* (Seki et al., 1983; Mimnagh et al., 1987) and a CRH innervation in *R. ridibunda* (Verhaert et al., 1984; Tonon et al., 1985) but other authors report that this innervation is scarce (Olivereau et al., 1987) or even absent (*R. catesbeiana*: Lederis, 1987; Gonzalez and Lederis, 1988). In *Xenopus* no direct innervation of the PI has been found by fibers containing CRH and TRH (Olivereau et al., 1987; present study: Chapter 7), and these transmitters are believed to reach the melanotropes by diffusion or via a portal system, after their release from the pituitary pars nervosa (Iturizza, 1969; Weatherhead, 1983; Jenks et al., 1993). Both in *Xenopus* (Chapter 7) and in the toad *Bufo arenarium* (Pasquier et al., 1980) injection of retrograde markers in the pars nervosa led to labeling of neurons in the Mg. In amphibians the Mg is the homologue of the mammalian paraventricular and supraoptic nuclei (Goos, 1978) and contains vasotocine- and mesotocine-positive cells (González and Smeets, 1992a) that project to the pars nervosa. Taking these data together, it

can be concluded that the Mg of *Xenopus*, and probably of amphibians in general, is not only involved in the neurohormonal release of vasotocin and mesotocin, but also stimulates α-MSH secretion from the melanotrope cells, by releasing CRH and TRH.

The question arises whether the Mg is the only stimulatory centre for melanotrope cell activity. It is known for a long time that animals in which the Mg has been lesioned do not become completely black on black background but retain a rather dark grey appearance (melanophore index: 3.5; Dierikcx, 1967), suggesting the necessity of the Mg for complete adaptation to a black background under natural conditions. When the neuro-intermediate lobe is completely denervated there is a massive release of α-MSH. Also *in vitro* neurointermediate lobes display a high and sustained "spontaneous" α-MSH release (Etkin and Sussman, 1961; Etkin, 1962a,b; Jørgensen and Larsen, 1963; Enemar and Falck, 1965; Hadley and Bower, 1976; Hadley and Hruby, 1977). Therefore, it seems that in addition to the Mg, a stimulatory mechanism within the neurointermediate lobe controls the release of α-MSH. Possibly, this mechanism involves the release of acetylcholine which stimulates α-MSH release from the melanotropes in an auto-excitatory way (F.J.C. van Strien, unpublished results).

Locus coeruleus

In Chapter 7 evidence is presented that the pars intermedia is innervated by neurons in the LC, as LC neurons become retrogradely labeled after injection with carbocyanine dyes into the pars intermedia. The same result were obtained in *Rana* and *Triturus* (Artero et al., 1994). Noradrenaline has been shown light immunocytochemically to be present in fibers in the *Xenopus* pars intermedia and in LC neurons (González and Smeets 1993). Pharmacological data suggest the presence of (nor)adrenergic receptors on the melanotrope cells (H.P. De Koning, personal communication) and superfusion experiments have shown a strong inhibitory activity of noradrenaline on α-MSH-release (Verburg-Van Kemenade 1986c). Therefore, it seems likely that LC neurons inhibit α-MSH secretion by releasing noradrenaline in the vicinity of the melanotrope cells. The way LC neurons deliver noradrenaline to the melanotrope cells is still unclear.

The dopamine in the fibers in the pars intermedia could in fact represent partly a noradrenergic innnervation as noradrenaline is synthesized from dopamine. However, up to now the demonstration of noradrenaline at the ultrastructural level in the pars intermedia has failed (*e.g.* C.A.F.M. Berghs, unpublished data). Since the vast majority of the synapses in the pars intermedia contain NPY, dopamine and GABA (Van Strien et al., 1991; De Rijk et al., 1990a,1992;) and do not seem to originate in the LC (see below ad suprachiasmatic nucleus), it may well be that the LC neurons release noradrenaline in or near the intermediate lobe in a non-synaptic, paracrine fashion.

Suprachiasmatic nucleus

The main emphasis in this thesis was to show the involvement of the suprachiasmatic nucleus in the light-dependent inhibition of melanotrope cell activity. Dierikcx observed that animals of which the Mg has been lesioned are still capable of paling their skin colour when put on a white background (Dierikcx, 1967), and he concluded that the inhibitory centre had to be situated in an area caudal to the Mg. The work of Dierikcx therefore does not rule out the SC as an inhibitory center as suggested by W.W. Douglas (Kongsamut et al., 1991).

Superfusion experiments have shown that NPY has a clearly inhibitory effect on α-MSH release (Verburg-Van Kemenade et al., 1987a). In Chapter 4 the distribution of NPY in the *Xenopus* brain and the hypophysis has been dealt with in detail, extending the data provided for *Xenopus laevis* (Lázár et al., 1993) and *Rana ridibunda* (Danger et al., 1985; Andersen et al., 1992; Lázár et al., 1993). It appears that NPY is present in a fiber network in the pars intermedia as well as in a number of hypothalamic nuclei. Applying *in situ* hybridization with a *Xenopus*-specific prepro-NPY-mRNA probe and immunocytochemistry with anti-NPY on pars intermedia from animals kept under different background light conditions, indications were obtained for a dynamic involvement of suprachiasmatic NPY-producing neurons (Chapter 5) in the inhibition of melanotrope cell activity. This idea was subsequently supported by administration of carbocyanine dyes to the fiber network innervating the pars intermedia. In *Xenopus* (Chapter 7) as well as in *Rana* (Artero et al., 1994)

retrograde labeling of suprachiasmatic neurons occurred after injection of these tracers into the pars intermedia.

A further confirmation of the idea that the SC controls melanotrope cell activity was derived from the study of neurotransmitter coexistence. Coexistence of NPY, DA and GABA has been shown at the ultrastructural level to occur in the synaptic contacts of the fiber network that innervates the *Xenopus* melanotrope cells (Van Strien et al., 1991; De Rijk et al., 1992), a situation which also occurs in the frog, *Rana ridibunda* (Tonon et al., 1992). This coexistence in synapses led to the search for the cell bodies of the neurons in the central nervous system. Combining the results concerning the distribution of NPY-producing neurons (Chapter 4 and 5) and the localization of TH-containing neurons (Chapters 1 and 2; González and Smeets 1991) it can be concluded that only three diencephalic nuclei are candidates for having neurons that contain both NPY and DA: the SC, the posterior tubercle (TP) and the posterior thalamic nucleus. Using the fluorescent markers fluorescein isothyocyanate (FITC) and Texas Red to stain immunocytochemically NPY and TH together in the same section (*i.e.* within the same neuronal cell body), a number of neurons in the SC were shown to produce both NPY and TH. Apparently, these neurons are the sites of origin of the synapses found in the pars intermedia to contact the melanotrope cells. Preliminary data indicate that the cells also express GABA (R. Tuinhof and A.O. de Graaf, unpublished data; A. Fasolo, personal communication).

In the SC the majority of the NPY-positive neurons do not seem to produce dopamine as they are immunocytochemically negative with the TH-antiserum. Nevertheless, all NPY-positive neurons show immunocytochemic changes and in their reaction to NPY RNA probes (*in situ* hybridization) with changes in background colour (Chapter 5). This would seem to indicate that not only the NPY-neurons coexpressing NPY and dopamine but also those expressing NPY alone, are involved in melanotrope cell control. The way the latter would act upon the melanotropes is unknown. Three possibilities may be taken into consideration. First, it may be that dopamine is present in all NPY-containing neurons but that the amount of TH in some NPY neurons is too low for immunocytochemical detection. Secondly, there may exist a dual inhibitory innervation of the *Xenopus* pars intermedia, one system innervating by NPY, GABA and DA, the other by NPY (and possibly GABA). A similar situation has

been proposed for the pars intermedia of *Rana esculenta*, where there would be two NPY-positive innervations, one originating from the preoptic area (using both NPY and GABA) and one from the infundibular area (containing only NPY; Tonon et al., 1992). However, this possibility is not supported by immuno-electron microscopic observations indicating that (nearly) all synaptic contacts in the pars intermedia of *Xenopus* contain DA (Van Strien et al., 1991; De Rijk et al., 1992). A third possibility is that the NPY-positive neurons lacking dopamine do not project to the pars intermedia but have other, light-dependent functions, such as serving as interneurons that control within the SC nucleus the activity of the NPY-neurons running to the hypophysis. In this case, it might be expected that they are the neurons that have been shown to be contacted by axons running from the optic tract (Chapter 7).

The developmental study also indicates that the SC is an important centre for regulation of the melanotrope cells. During the embryonic stages 39-41 the neurointermediate primordium reaches its final position caudoventral of the infundibulum (Nyholm, 1977; Nyholm and Doerr-Schott, 1977; Eagleson et al., 1986; Verburg-Van Kemenade, 1984) and the melanotrope cells start to produce and secrete α-MSH (Nyholm and Doerr-Schott, 1977). At the same time a dopaminergic innervation within the pars intermedia is present (Terlou and Van Straaten, 1973), the animal becomes capable of adjusting its skin colour, and its melanotrope cells become sensitive to neural inhibition (Verburg-Van Kemenade, 1984). The development of catecholamine- and NPY-containing systems in the *Xenopus* brain has been described in detail in Chapters 2 and 4. This description demonstrates that the putative inhibitory brain centers, the SC and the LC, start to express their activity around the same time, *i.e.* at stages 39-41.

Conclusions and perspectives

The regulation of the activity of the melanotrope cells appears to be very complex. The cells seem to be controlled by a dual (SC and LC), partly multiple, inhibitory system (LC producing NA, SC producing NPY, DA and GABA) and by a multiple stimulatory system (Mg producing CRH and TRH). The role of the SC seems obvious: it produces and releases NPY, GABA and DA, which inhibit α-MSH release from the melanotrope cells. The fact that background light

intensity affects the activity of NPY-producing cells in the SC and that these cells receive optic input, strongly suggests that NPY (and probably GABA and DA as well) transmit light information perceived by the eyes toward the melanotropes. The physiological significance of the inhibitory control of the LC and the stimulatory control by the Mg is not known. Probably, they are not (only) involved in adaptation to environmental light conditions but (also) transmit other environmental information which might affect melanotrope cell activity, such as handling stress and temperature (Waring, 1963; Terlou et al., 1974). The present results raise a variety of new questions worth investigating. This chapter will be concluded by considering some of these questions.

Firstly, it may be questioned why the SC would express three different inhibitors to control melanotrope cell activity. The inhibition by GABA and NPY of the melanotrope cells appears to differ both in duration and in strength of action, the effect of NPY being the slowest and having the longest duration. A possibility is that GABA, found in the electron lucent vesicles, would be utilized for short-term inhibition, while NPY (and dopamine), present in electron-dense vesicles, would be released during long-term adaptation (Leenders et al., 1993).

Secondly, in mammals the SC is known to play an important role as a circadian clock, governing various physiological rhythms (*e.g.* Rusak and Zucker, 1979, 1990; Moore and Card, 1990). Therefore, the possibility that in *Xenopus* SC neurons control the melanotropes in a circadian fashion deserves attention. Diurnal rhythms have been reported in *Xenopus* with respect to interrenal activity (Thurmond et al., 1986a,b; Lange and Hanke, 1988), retinal melatonin levels (Cahill et al., 1991; Cahill and Besharse, 1993) and the state of pigment dispersion in skin melanophores (Waring, 1963). Involvement of the SC in the regulation of these rhythms in amphibians has not yet been shown.

A third question concerns the complex neuronal circuitry that is involved in the regulation of the process of background adaptation. In this thesis neurons that directly influence this activity of the melanotrope cells have been located in three different nuclei, the Mg, the LC and the SC. The next step in a better understanding of the complex neural pathway involved in background adaptatation is knowledge about the neuronal regulation of these neurons. Tract tracing studies will be needed to show the origin of this next order of neurons. Their nature, the neurochemical messengers they produce and their connectivities will have to be shown by dual labeling immunocytochemical

techniques. Research on these issues has been started in our department (R. Ubink).

Finally, an intriguing phenomenon is the plasticity of the innervation of the melanotrope cells. Preliminary results indicate that in animals that are adapting to a white background the number of fibers that innervate the melanotrope cells increases and the synaptic contacts with the melanotropes increase in size as compared to black-adapted toads (C.A.F.M. Berghs). Thus, light-mediated activation of the SC might become a suitable model for studying the structural aspects of neuronal plasticity in the relation to a well-defined physiological process.

SAMENVATTING

Om te kunnen overleven moet een dierlijk organisme informatie opnemen uit de omgeving. Na verwerking, kan deze informatie gebruikt worden om het functioneren van verschillende orgaansystemen te sturen en onderling te coördineren waardoor het dier in staat is zich aan te passen aan veranderende omstandigheden in zijn omgeving. Het zenuwstelsel en het endocriene systeem spelen een belangrijke rol in de overdracht van informatie en de handhaving van de inwendige homeostase. Externe informatie wordt door zintuigen omgezet in electrische signalen die doorgegeven worden aan het centrale zenuwstelsel. Na verwerking van de informatie treedt er een direct perifeer effect op (bijvoorbeeeld stimulatie van spieren, klieren of chromatoforen). Een belangrijk doelwit van het zenuwstelsel is het endocriene systeem. Dit systeem geeft speciale boodschappers, de hormonen, af aan het bloed. Via de circulatie worden de hormonen door het lichaam getransporteerd waardoor ze elders in het lichaam hun effect teweeg kunnen brengen. De regulatie van hormonale activiteit door het zenuwstelsel vindt in zeer belangrijke mate plaats door interactie tussen de hypothalamus en de hypofyse. Deze regulatie is vooral van belang bij de sturing van lange termijn-processen (bijvoorbeeld groei, voortplanting en ionen- en water-huishouding).

Nerveuze regulatie van endocriene activiteit is het onderwerp van dit proefschrift. Als onderzoek model is gekozen voor de neurale controle van het complexe neuro-endocriene regulatieproces van achtergrondadaptatie door de Zuidafrikaanse klauwpad, *Xenopus laevis*. Dit dier is in staat om de huidskleur aan te passen aan de lichtintensiteit van de ondergrond. De optische informatie wordt opgevangen door de retina en omgezet in electrische signalen die via de oogzenuw doorgegeven worden aan het centrale zenuwstelsel. Na bewerking in de hersenen wordt het signaal verder geleid, vermoedelijk naar de hypothalamus van waaruit dan een opdracht wordt verstuurd naar het middelste deel van de hypofyse, de pars intermedia. Hier, in de pars intermedia, bevinden zich de melanotrope cellen die in staat zijn het α-melanoforen-stimulerend hormoon (α-MSH) te synthetiseren en af te geven aan de bloedbaan. Dit α-MSH stimuleert in huidcellen (melanoforen) een dispersie van het zwarte pigment melanine waardoor de huid van de pad donker wordt.

De aanmaak en afgifte van α-MSH worden gereguleerd vanuit het centrale zenuwstelsel door neurochemische boodschappers. Eerder is aangetoond dat

neuropeptide Y (NPY), γ-aminoboterzuur (GABA), dopamine (DA) en noradrenaline een remmende werking hebben op de aanmaak en afgifte van α-MSH. Met immunocytochemische methoden zijn deze inhibitoire neurotransmitters lichtmicroscopisch aangetoond in een axonaal netwerk dat gelegen is tussen de melanotrope cellen. Op ultrastructureel niveau blijkt dit netwerk synapsen te vormen met de melanotrope cellen. In deze synapsen komen NPY, GABA en DA samen voor (coëxistentie). Stimulatie van de melanotrope cellen wordt teweeggebracht door het corticotropine releasing-hormone (CRH) en het thyrotropine releasing-hormone (TRH). Deze secretagogen worden afgegeven vanuit de pars nervosa van de hypofyse en werken via diffusie op de melanotrope cellen in de pars intermedia.

Dit proefschrift beoogt een antwoord te geven op de vraag welke hersencentra de neurotransmitters die de melanotrope cellen van *Xenopus laevis* reguleren, produceren.

In **hoofdstuk 1** wordt de distributie beschreven van dopamine-bevattende neuronen en vezels in het centrale zenuwstelsel van *Xenopus* in de volwassen situatie. In het diencephalon zijn dopamine-houdende perikarya aangetroffen in de voorste preoptische-, suprachiasmatische- en achterste thalame kernen, in het tuberculum posterior en in het paraventriculaire orgaan.

Het ontstaan van deze hersencentra gedurende de embryonale en larvale ontwikkeling staat centraal in **hoofdstuk 2**. Het tuberculum posterior, het paraventriculaire orgaan en de suprachiasmaticus komen al embryonaal (stadium 39-40) tot expressie, terwijl de achterste thalame kern in de larvale periode rond stadium 53 en de voorste preoptische kern pas vlak voor de metamorphose (stadium 59) ontstaat.

In **hoofdstuk 3** worden het tuberculum posterior en de suprachiasmatische kern voorgesteld als mogelijke locaties in de hypothalamus waar de inhibitie van de pars intermedia vandaan komt. Deze twee kerngebieden bevatten dopamine-producerende neuronen. Bovendien worden in deze kernen neuronen gevonden die NPY of GABA bevatten, waardoor een coëxistentie van de drie neurotransmitters in één neuron hier mogelijk is.

Het voorkomen van NPY-producerende cellen en vezels in volwassen en zich ontwikkelende *Xenopus* wordt beschreven in **hoofdstuk 4**. In volwassen padden worden in het diencephalon NPY-positieve neuronen gevonden in de suprachiasmatische (39-41), ventromediale en achterste thalame (43/44) en

ventraal infundibulaire kernen (43-44) en in het tuberculum posterior (43/44). Op grond van de distributie van DA en NPY komen de suprachiasmatische en achterste thalame kernen en het tuberculum posterior in aanmerking als plaats van oorsprong van de innervatie van de pars intermedia. Rond stadium 39/40 heeft de hypofyse zijn uiteindelijke plaats bereikt, begint de α-MSH produktie op gang te komen en is de larve in staat om zijn huidskleur aan te passen aan die van zijn omgeving. Rond dit stadium is de suprachiasmatische kern de enige die zowel DA als NPY tot expressie brengt.

In **hoofdstuk 5** wordt de veranderende activiteit van NPY-producerende diencephale neuronen onder verschillende achtergrondcondities, beschreven. Met behulp van een antiserum tegen NPY werden suprachiasmatische neuronen aangekleurd in wit-geadapteerde dieren. In dieren die aangepast waren aan een zwarte ondergrond was de immunoreactie in de suprachiasmatische kern negatief. Dit was niet alleen het geval voor de NPY-immunocytochemie maar ook met *in situ* hybridisatie werd met een *Xenopus* specifieke prepro-mRNA NPY-probe alleen in de suprachiasmaticus van wit-geadapteerde dieren een positief hybridisatie signaal aangetroffen. Quantitatieve beeldanalyse heeft aangetoond dat dit effect specifiek is voor de NPY-neuronen in de suprachiasmaticus.

Met behulp van immunofluorescentie met verschillende antilichamen en fluorochromen is het gezamenlijk voorkomen van DA en NPY in suprachiasmatische neuronen onderzocht. In **hoofdstuk 6** wordt de aanwezigheid van drie verschillende suprachiasmatische populaties beschreven, een dopaminerge, een NPY-bevattende en een populatie waar de neuronen beide transmitters tegelijkertijd tot expressie brengen.

Retrograde en anterograde tracers zijn toegepast om de verbindingen van de oogzenuw met de hersenen en van de hersenen met de hypofyse aan te tonen. In **hoofdstuk 7** wordt beschreven dat na het inspuiten van carbocyanine kleurstoffen in de pars intermedia retrograad neuronen aankleurden in de locus coeruleus en de suprachiasmatische kern. Na vulling van de pars nervosa werden gelabelde neuronen teruggevonden in de magnocellulaire kern. Anterograde markers die werden ingespoten in de oogzenuw, gaven behalve een projectie naar de optische gebieden ook een innervatie van de suprachiasmatische kern te zien, waarbij varicositeiten van optische vezels lijken te eindigen op NPY-positieve neuronen.

De resultaten die zijn beschreven in de voorgaande zeven hoofdstukken hebben geleid tot de vorming van een model van de innervatie van de pars intermedia. Drie gebieden in de hersenen van *Xenopus* lijken een belangrijke rol te spelen in de neuro-endocriene regulatie van achtergrondadaptatie. Van rostraal naar caudaal zijn dit de hypothalame magnocellulaire kern, de suprachiasmatische kern en de locus coeruleus.

De magnocellulaire kern is de oorsprong van de secretagogen CRH en TRH die worden afgegeven in de pars nervosa. Injectie van tracers in de pars nervosa veroorzaakt een uitgebreide retrograde labeling van neuronen in de hypothalame magnocellulaire kern. Na te zijn afgegeven in de pars nervosa bereiken CRH en TRH waarschijnlijk via diffusie de melanotrope cellen in de pars intermedia en hebben een stimulerend effect op de secretie van α-MSH.

De suprachiasmatische kern speelt een belangrijke rol in de inhibitie van de melanotrope cellen. Er zijn neuronen gevonden in het caudolaterale deel van de SC, tegen het chiasma aan, die in staat zijn zowel DA als NPY te produceren. De mogelijke optische innervatie van NPY-positieve neuronen in de SC wijst erop dat deze inhibitie van de melanotrope cellen lichtafhankelijk is.

Een derde, extrahypothalame, kern die mogelijk betrokken is bij de regulatie van de melanotrope cellen is de locus coeruleus. De locus coeruleus bevat noradrenerge neuronen, er is een noradrenaline-positief netwerk in de pars intermedia en er is in superfusie experimenten aangetoond dat noradrenaline een inhibitoir effect heeft op de secretie van α-MSH. Deze directe noradrenerge innervatie van de pars intermedia is mogelijkerwijs betrokken bij regulatie van door stress geïnduceerde kleurveranderingen van de huid.

ABBREVIATIONS

A	adrenaline
ac	anterior commissure
Acc	nucleus accumbens
Aob	accessory olfactory bulb
AP	area postrema
Apl	lateral amygdala
Apm	medial amygdala
Av	anteroventral tegmental nucleus
CA	catecholamine
Cb	cerebellum
cc	central canal
CRH	corticotropin releasing hormone
CSF	cerebrospinal fluid
DAB	3,3'-diaminobenzidine
DA	dopamine
DB	diagonal band of Broca
GABA	γ-amino butyric acid
gcl	ganglion cell layer of the retina
gl	glomerular layer of the olfactory bulb
gr	granular cell layer of the olfactory bulb
H	habenula
il	intermediate lobe of the hypophysis
inl	inner nuclear layer of the retina
Ip	interpeduncular nucleus
ipl	inner plexiform layer of the retina
IR	infundibular recess
Is	isthmic nucleus
LC	locus coeruleus
LFB	lateral forebrain bundle
LL	lateral line nucleus
Lp	lateral pallium
Ls	lateral septum
ml	mitral cell layer of the olfactory bulb
Mp	medial pallium
Ms	medial septum
nVIII	octaval nerve
α-MSH	α-melanophore stimulating hormone
ME	median eminence
NA	noradrenaline
nl	neural lobe of the hypophysis

NPY	neuropeptide Y
NPv	nucleus of the paraventricular organ
ob	olfactory bulb
OC	optic chiasm
Ols	superior olivary nucleus
onl	olfactory nerve layer
opl	outer plexiform layer of the retina
P	posterior thalamic nucleus
PAP	peroxidase antiperoxidase
pc	posterior commissure
Pd	posterodorsal tegmental nucleus
PD	hypophysis, pars distalis
PI	hypophysis, pars intermedia
PN	hypophysis, pars nervosa
POa	anterior preoptic nucleus
POMC	proopiomelanocortin
Ri	inferior reticular nucleus
Rm	medial reticular nucleus
S	septum
SC	suprachiasmatic nucleus
sol	solitary tract
Str	striatum
TBS	Tris-buffered saline
TBS-TX	Tris-buffered saline with triton
Tect	mesencephalic tectum
TH	tyrosine hydroxylase
Thd	dorsal thalamus
Thv	ventral thalamus
Tor	torus semicircularis
TP	posterior tubercle
TRH	thyrotropin releasing hormone
v	ventricle
VH	ventral hypothalamic nucleus
VM	ventromedial thalamic nucleus
VTA	ventral tegmental area

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DANKWOORD / ACKNOWLEDGEMENTS

Er moet enige tijd voorbij gaan voordat je een deel van je verleden gaat romantiseren, zoals dienst, hoewel er ook daar wel minder leuke perioden zijn geweest. Daar kwam het einde voor mij te snel en was ik graag nog wat langer gebleven. Voor Nijmegen geldt een beetje hetzelfde, aan de ene kant ben ik blij dat ik ergens anders opnieuw ga beginnen, ik hoop minder eenzaam, aan de andere kant zal ik de sfeer binnen de vakgroep missen. Binnenkort (volgend jaar?), zal er waarschijnlijk ook niets beter zijn dan Nijmegen, en ik hoor jullie al denken, die loopt straks ergens anders door de gangen te fluisteren, "dit huppeldepup instituut .....".

De hulp en het optimisme van collega's en studenten is onontbeerlijk geweest om voldoende materiaal te verzamelen om dit proefschrift succesvol af te ronden. De goede samenwerking van de afdeling Cellulaire Dierfysiologie van Eric Roubos, met de vakgroepen Anatomie aan de KUN en de VU, en buitenlandse Biologische laboratoria van de universiteiten van Rouen, Turijn, Córdoba en Madrid is van onschatbare waarde geweest.

Van de mensen die bij de Vakgroep Anatomie en Embryologie van de Vrije Universiteit in Amsterdam werkzaam zijn wil ik Floris Wouterlood voor de infectie met het anatomie virus en Peter Goede voor het bijbrengen van de neuroanatomische technieken bedanken; jullie hebben de aanleiding gevormd tot mijn verblijf aan de KUN. De overgang van het werken aan de hersenen van de rat bij jullie en die van de Zuid Afrikaanse klauwpad tijdens mijn KUN-periode is enorm geweest, maar ik heb steeds terug kunnen vallen op jullie vakgroep, en voor wat betreft kikkers met name Wil Smeets. Wil, tegelijk met jou, wil ik Agustin González (Departamento de Biologia Animale, Universidad Complutense, Madrid) heel erg bedanken voor alle steun, constructieve discussies en een bijzonder fijne samenwerking die heeft geleid tot een aantal zeer fraaie publicaties. Agustin, muchas gracias por el apoyo, las discusiones constructivas y por la agradable colaboración que ha conducida a unas publicaciones excelentes.

Ik dank binnen de Vakgroep Anatomie en Embryologie van de KUN, Hans ten Donkelaar, Roelie de Boer-van Huizen en Henk Joosten voor het altijd aanwezig zijn om me met raad en daad bij te staan. I like to thank Cristina Artero, Aldo Fasolo, Fosca Franzoni (Dipartimento di Biologia Animale,

Università di Torino, Italy), Hubert Vaudry (Laboratoire d'Endocrinologie Moleculaire, Université de Rouen, France), Paco Gracia Navarro (Departamento de Biologia Celular, Universidad de Córdoba, Spain) for the pleasant cooperation, mutual publications and for motivating your students to participate in the ERASMUS exchange programme. I enjoyed very much to work with these students and am grateful for their contribution to my thesis.

Nijmegen, de groep van Eric Roubos, Cellulaire Dierfysiologie is wel een hele ommekeer geweest, en ik ben behalve op het werk, hier niet erg gelukkig geweest. Qua werk was het ook een hele verandering ten opzichte van Amsterdam. Het fraaie stevige hersenweefsel van de rat werd verwisseld voor het vormloze glibberige bultje slijm van de klauwpad *Xenopus*. Eric je hebt dit, voor het tekenen van mijn contract, wijselijk verzwegen of er gewoon niet aan gedacht het te zeggen, ik weet dan ook niet of ik het nog eens over zou doen. Aan de mensen van jouw groep, maar ook die van Sjoerd Wendelaar Bonga en Gerard Martens heeft het zeker niet gelegen. Binnen de vakgroep Experimentele Dierkunde, heb ik mogen werken met een hele leuke groep mensen in een heel plezierige informele sfeer, die voor mij, komende van een medische faculteit, toch wel wat vreemd was. Eric Roubos, Bruce Jenks, Hans Leenders, Evelien de Rijk, Harry de Koning, Wim Scheenen, Frank van Strien, Walter van Heumen, Stanny Berghs, Will Spooren, Jacco Lieste, Corinne Dotman, Ruud Ubink en Werner Koopman, de vier jaar die ik hier met jullie heb mogen samenwerken zijn omgevlogen, het is alsof het gisteren was dat ik voor het eerst voor het UL stond om de vakgroep Zoölogie te zoeken. Net nu ik een beetje tot de inboedel ga behoren en bekende vreemde gezichten in de lift je 's ochtends (nou iets later in de morgen) groeten, is het voorbij. Ik heb met veel plezier met jullie samengewerkt en met name de sessies en discussies in de koffiekamer erg gewaardeerd, hoewel ik wel het idee had dat de pot vaak net leeg was als ik langs kwam. De kantine vond ik minder geslaagd, een ideeën-bus zou uitkomst kunnen bieden, maar ik zie wat dat betreft de toekomst van de frikandel somber in. Ik kom vast terug om jullie op te zoeken, in Amsterdam noemden ze me al Heintje Davids nadat ik vertrokken was, en daar heb ik niet zo lang gezeten, dus pas maar op.

Tony Coenen, Ron Engels, Maarten van Riel, Jelle Eygensteyn en Peter Cruijsen, ik dank jullie voor alle technische hulp die jullie altijd bereid waren te geven. Tony, ik heb altijd graag beneden even met je zitten kletsen, een sigaretje

paffen en zo tussendoor even van alles regelen en oplossen wat maar met immunocytochemie te maken had, je wist altijd wel wat nuttigs uit een of andere vriezer tevoorschijn te toveren of na een gemaakt foutje snel een bruikbaar lapmiddel te bedenken. Het meest erkentelijk ben ik je vanwege het feit dat je voor mij één uitzondering hebt willen maken en op mijn promotie, samen met Stanny, paranimf wilt zijn.

Else Schaberg, toen ik er aan het begin van vorig jaar schoon genoeg van had en alvast wilde beginnen met verhuizen heb jij dat tegen weten te houden, achteraf jammer want nu moet het alsnog in één keer, maar ik ben blij dat jij en Herman me hebben weten op te beuren.

Ik wens jullie allemaal zeer veel succes met je werk en voor wat betreft de AIO's/OIO's van Zoölogie hoop ik in de toekomst een exemplaar van jullie proefschrift te krijgen zodat ik een reden heb weer eens naar mijn oude lab terug te gaan. Alle botanen in het sousterrain, zoölogen / medewerkers van de tweede, derde en vierde, ik zal jullie missen.

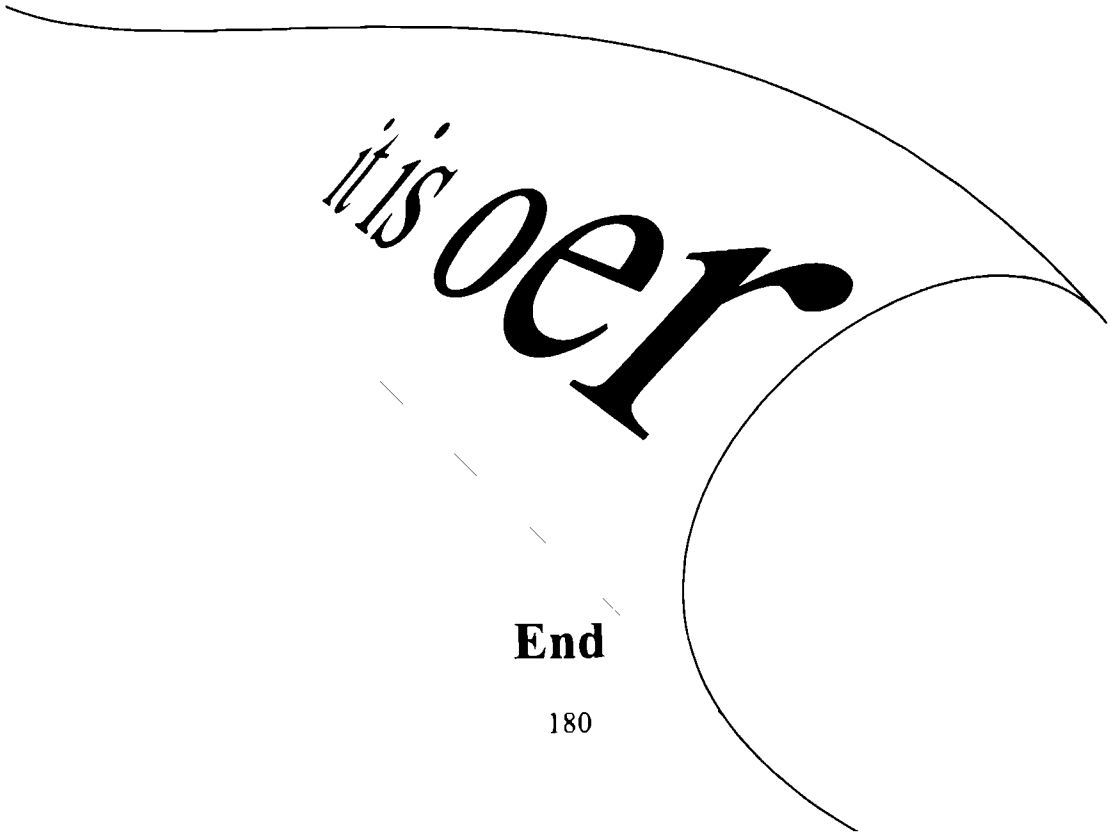
De studenten die bij mij een hoofd- of bij-vakstage hebben gelopen, Carola Michielsen, Jan-Willem Brandt, Gabriel Beckers, Anneloes Dirks, Renate Ebbers, Aniek de Graaf en Ronnie Wismans (die als vrijwilligster heel veel werk heeft gedaan), ik dank jullie allemaal voor je inzet en hulp, want hoewel het misschien niet altijd zichtbaar is geworden heeft jullie werk veel bijgedragen aan de totstandkoming van mijn proefschrift.

The three foreign students who had to bare the burden of my supervision and contributed to this thesis. Françoise Laurent, tu as trouvé la source, pour moi tu restes toujours la première, merci pour tous, tgm. José Carlos Garrido Garcia, no olvidaré nunca las comidas 'shoarma', particularmente tus cuentas sobre la limpieza de los dientes y de la lengua para librarte del olor de ajos. ¡ Muchas gracias! Cristiana Atzori, peccato che la tua ultima settimana sia stata triste. Spero che sia stata a causa della tua partenza! La tua tesina è stata la più divertente, grazie di tutto.

My fellow foreigners in Nijmegen, it is unfortunately impossible to change your past. Françoise Laurent, Claire, Bertrand, Anne, Laurence, Ludo, Cristelle, José-Carlos, Marco, Stefania, Barbara, Cristiana, Monica, Domenico, Sebastien, Kiki, Aude, Maia and Françoise Collin, you all made living here, in this terrible village near a ditch, a little bit less lonely and more bearable. I thank you all for the pleasant evenings eating, barbecuing or drinking in town or at

somebodies home. For the same reason I thank my colleagues especially, Stanny, Else/Herman, Titti, Wim, Harry, Anne Haaijman, Bruce/Ann, Eric/Ellen, Hans/Toos, Wil/Marina and Frank/Manon. I thank you all for having been there. Kiki I like to finish with your answer to the question of the Trivial Pursuit game, *What is the final word of the Bible?* I had the same answer and in the case of my thesis this is really the

End



End

Curriculum vitae

De promovendus is AIO geweest op de Afdeling Cellulaire Dierfysiologie van Prof. Dr Eric W. Roubos van 1 november 1990 tot 1 november 1994.